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THE INFERENCE OF INTRACELLULAR ENZYMATIC PROPERTIES FROM KINETIC DATA OBTAINED ON LIVING CELLS

I. SOME KINETIC CONSIDERATIONS REGARDING AN ENZYME ENCLOSED BY A DIFFUSION BARRIER

JAY BOYD BEST [1]

The Department of Physiology and The Committee on Behavioral Sciences, The University of Chicago

ONE FIGURE

It is the purpose of this paper and a companion one (Best, '55) to demonstrate a method by means of which some of the properties of enzymes in living cells can be investigated and the membrane permeability of the cell to their substrates measured.

Because of the limitations imposed by most of the methods hitherto available for the technical execution of the measurement of the permeability constant of cells for substrates that undergo metabolic conversion by those cells, few such values are available. A resume of the more important of these methods is given in the companion paper. The considerations on which these methods are based include the assumption that the test substance is, for all practical purposes, conserved during the course of the experiment, thereby precluding their use for the measurement of the permeability for test substances that are metabolized at an appreciable rate by the cell under investigation.

If one wishes to measure the membrane permeability of a cell to a substance that undergoes metabolic conversion at a

[1] This work was done in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology of the University of Chicago. The writer was a United States Public Health Service Fellow at the time.

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rate that is appreciable in comparison to the rate of entry, it is necessary to take the rate of conversion into consideration.

The differential equations [2] of the material balance for a substance entering the cells of a suspension can be written as

$$V\, dS_i/dt = \beta \cdot (S - S_i) - Q(S_i) \qquad (1)$$

$$dS/dt = -N\beta \cdot (S - S_i) \qquad (2)$$

in which N is the number of cells per unit volume, S the concentration of the substance (substrate) in the medium, S_i its concentration in the cell, V the volume of the cell, and $Q(S_i)$ the rate at which the substance is consumed per cell. $Q(S_i)$ will be a function of S_i. In order to measure the value of the coefficient β (which under certain circumstances will turn out to be just the permeability constant multiplied by the surface area of the cell) one must have knowledge of the actual value of S_i or eliminate it from the pair of differential equations to obtain an expression that does not contain S_i. In general the value of S_i is difficult to measure directly since the process must be stopped in some manner, the cells separated from the medium and disrupted for execution of a chemical analysis. All of these procedures require time during which S_i may be changing by diffusion or metabolic conversion. Thus, if the time required to stop the process is Δt, then the error incurred in the measured value of S_i would be

$$\Delta S_i = \frac{1}{V} \int_t^{t+\Delta t} (\beta \cdot (S(t') - S_i(t')) - Q(t', S_i))\, dt'.$$

One seldom knows the value of this integral.

The alternative method is to assume a realistic function form for Q that will describe the behavior of Q as a function of S_i over the range of S_i likely to be encountered under the experimental conditions in which the measurement is to be carried out. The variable S_i can then, in principle, be eliminated from the two first order differential equations to obtain a second order differential equation in S alone. One can divide

[2] For a discussion of the assumptions involved in writing equation (1) see Rashevsky (Chap. I, 1948) and Hearon (1953, I and II).

kinetic analyses of this type into two main classes each of which, in practice, have their virtues and defects. One of these is the time dependent formulation in which one attempts to deduce the values of β and the other parameters defining Q by an analysis of the transient behavior of the rate of disappearance of the test substance into the cell, e.g. Weichherz ('29, '31). The second method is to invoke a steady or quasi-steady state condition by means of which the differential equations involving the derivatives of the concentrations with respect to time are reduced to ordinary algebraic equations, e.g. Rashevsky (Chap. III, '48), Landahl ('39, '48), Hearon ('49a, b). The time dependent formulation has the advantage of permitting parameters, which may be experimentally inseparable under the conditions of the steady state formulation, to be separately evaluated, but has the severe disadvantage that all too frequently an adequate functional form for Q leads to a non-linear differential equation in which mathematical intractibility is the rule rather than the exception. The steady or quasi-steady state formulations can frequently be shown to approximate the experimental conditions very closely, even when the external substrate concentration may be changing, and permit one to utilize a more realistic expression for the concentration dependence of the rate of metabolic conversion with hope of obtaining a useful answer.

It is perhaps obvious, but important enough to point out, that the value of β obtained by an analysis of a given set of experimental data (or rates of disappearance for various concentrations of substrate in the external medium) will depend upon the formal model, i.e. functional form of $Q(S_i)$, chosen to express the rate of conversion of the test substance inside the cell. One must, therefore, consider the measurement of the membrane permeability for a substance that undergoes catalytic conversion in the cell as being inseparable from the problem of experimental measurement of the formal kinetic parameters of the intracellular catalysis.

In this paper a theoretical model will be set up in which the functional form of $Q(S_i)$ is assumed to be that given by the

Michaelis and Menten rate expression for enzymatically catalyzed processes. Three possible procedures for application of this model to the analysis of experimental data are discussed. One of these, the non-linear regression method, is much the preferable and is used in the companion paper (Best, '55) for the computation of β and the parameters of $Q(S_i)$ for three sets of experimental data on baker's yeast. The results of the experiments described in the companion paper will be used in this paper to investigate the extent to which some of the more important assumptions of the model are fulfilled.

A. The Michaelis and Menten enzyme inside a diffusion barrier. Consider the situation in which a substrate that is being consumed by a cell must diffuse past a barrier to get to the enzyme that catalyzes its first metabolic conversion stage. In the case at hand it will be assumed that the manner of catalysis of the conversion is adequately described by the Michaelis and Menten formulation for enzymatic reactions.

In addition to those symbols which have already been defined the following notation will be used in the subsequent discussion:

V_{mc} = Michaelis V_m expressed on a "per cell" instead of "per unit volume" basis.
K = Michaelis constant of the enzyme,
A = cell surface area,
h = membrane permeability,
v_c' = rate of disappearance of the substrate per cell.

For a suspension of cells in a well stirred medium one can, without serious error, assume the substrate concentration immediately outside the cell membrane to be that given by chemical analysis of a macroscopic sample of the medium.

Assume that the rate of entry of the substrate into the cell can be expressed as

$$v_c' = \beta \cdot (S - S_i) \qquad (3)$$

where β is some constant that is characteristic of a particular type of cell for a given substance. If there are no external or internal concentration gradients then β will turn out to be just Ah. In the course of this derivation it will be assumed that

the internal gradients are negligible. In a subsequent portion of the paper it will be demonstrated that this assumption is justified in the case of some experiments on baker's yeast to which the formulation was applied.

The popular form of the Michaelis and Menten model for enzymatic reactions assumes a situation that may be described schematically in the following way

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products} \qquad (4)$$

in which S represents the substrate, E the free enzyme, ES the enzyme substrate complex, and k_1, k_{-1}, and k_2 the rate constants for the various processes indicated. The rate of conversion, v, by such a system is given by the expression

$$v = k_2 E_o \cdot (S)/(S + (k_{-1} + k_2)/k_1) \qquad (5)$$

where $E_o = (E) + (ES)$. In reality this model, proposed by Briggs and Haldane ('25), goes beyond that set forth by Michaelis and Menten ('13). Michaelis and Menten postulated that

$$K = (E)(S)/(ES) \qquad (6)$$

and seem to have viewed the constant K as though it were an equilibrium constant.

By postulating (6) and the relations

$$v = k_2 \cdot (ES)$$

$$E_o = (E) + (ES)$$

they obtained the rectangular hyperbolic relation

$$v = V_m \cdot S/(S + K). \qquad (7)$$

The equation shown in (7) has been applied to a number of experiments on a considerable number of different enzymes and found to describe the experimental results in quite a satisfactory manner. Apparently, however, Michaelis and Menten did not recognize that K was not a true equilibrium constant and that at least one step in the catalytic process

must be irreversible (Haldane, '30) to obtain the hyperbolic form given by equation (7).

Since Michaelis and Menten knew, in their original experiments, that large concentrations of fructose and glucose can inhibit invertase, their experiments were conducted so that the initial rates of hydrolysis were observed. Thus, except in their experiments designed to measure the competitive inhibition of the enzyme by the hydrolytic products of the reaction, the condition of an irreversible step was automatically fulfilled. The recognition that K in (7) is not an equilibrium constant but instead

$$K = (k_{-1} + k_2)/k_1$$

seems to have first been clearly expressed by Briggs and Haldane ('25) and by Haldane ('30) from the expression for v when all the steps are reversible.

Hearon ('52) has demonstrated that in many cases of catalysis of the transformation of a substrate by a complex multi-enzyme system it is possible to obtain concentration rate relation identical in form to equation (7) but in which the parameters V_m and K are not comprised solely of the rate constants of any single enzyme in the system. He prescribes the procedure by which, in principle, such a complex system could be analyzed by the systematic collection of these parameters for various substrates and intermediates introduced into the system.

For the most part, in this paper, it will not be necessary to interpret the detailed mechanism of the catalysis although in certain of the applications to which it may be put an interpretation will be necessary. For the formal analysis of the data for the evaluation of β, K, and V_{me} it is only necessary to assume that the rate dependence on the concentration of substrate in the immediate neighborhood of the catalyst is given by a function having the form of equation (7).

The rate v of equation (7) is usually expressed in terms of the amount of substrate converted per unit volume per unit time. If, however, v were expressed in terms of substrate

converted per cell, then V_m would become V_{mc} and, according to equation (5), would be directly proportional to the amount of enzyme per cell. With the assumption of no appreciable concentration gradients inside the cell then one can write

$$v_c = S_i \cdot V_{mc}/(S_i + K) \tag{8}$$

in which S_i is the internal substrate concentration at the site of the enzyme and v_c is the rate of catalytic conversion of the substrate per cell.

If one assumes that the rate of conversion by the enzyme is equal to the rate of penetration of the substrate into the cell, then

$$v_c = v_c'$$

and v_c becomes the rate of disappearance per cell of S from the external medium. Equation (3) can be readily solved for S_i to give

$$S_i = S - v_c/\beta. \tag{9}$$

If S is changing with time but the relation between S and S_i given by equation (9) is maintained for all practical purposes the system will be said to be in a *quasi-steady state.* Equation (9) can be substituted into equation (8) to give [3]

$$v_c = \frac{V_{mc}(S - v_c/\beta)}{(S - v_c/\beta) + K} \tag{10}$$

By simple algebraic rearrangement of equation (10) one obtains the relation

$$V_{mc} - v_c = K/(S/v_c - 1/\beta). \tag{11}$$

Equations (10) and (11) do not contain the quantity S_i which is technically difficult to measure. In the application of these equations it will not be necessary to impose a condition as stringent as the steady state. Since equation (9) was the actual result of the steady state condition it is really only necessary that the quasi-steady state condition be fulfilled. Thus S and S_i may be allowed to change with time provided that they effectively maintain the relationship between them given by (9).

[3] Equation (10), except for the units in which the rate is expressed, is the same as that obtained by Hearon (page 85, 1949b).

B. Evaluation of the parameters V_{mc}, K, *and* β *by the graphical method.* This method, although, in the writer's opinion, not as good for the evaluation of V_{mc}, K, and $1/\beta$ as the non-linear regression method to be described later, will be presented because of its interest as an alternative device for the analysis of a set of data and because it is computationally less laborious. It can also be used to elaborate, qualitatively, some discrepancies from the assumed reaction model that may not be obvious from the non-linear regression method.

One can simplify equation (11) by introducing the new variable

$$x = S/v_c \tag{12}$$

which gives

$$V_{mc} - v_c = K/(x - 1/\beta). \tag{13}$$

Differentiation of equation (13) with respect to x yields

$$dv_c/dx = K/(x - 1/\beta)^2 \tag{14}$$

or that

$$(dv_c/dx)^{\frac{1}{2}}/K^{\frac{1}{2}} = 1/(x - 1/\beta). \tag{15}$$

If one substitutes (15) back into (13) then one obtains

$$v_c = V_{mc} - \sqrt{K}\,(dv_c/dx)^{\frac{1}{2}}. \tag{16}$$

Expression (16) can readily be seen to be a linear equation such that if one plots $(dv_c/dx)^{\frac{1}{2}}$ as abscissa and v_c as ordinate one obtains a linear plot of slope $-\sqrt{K}$ and intercept V_{mc}.

An interesting feature of this plot is that if dv_c/dS becomes zero for a finite value of S then dv_c/dx becomes negative and its square root an imaginary number. This situation will occur in the neighborhood of a rate maximum such as that obtained when an excess of substrate acts as an inhibitor to the catalyst. Of course it is impossible to obtain a rate maximum for finite S from a Michaelis and Menten catalyst and equation (16) will not give a real solution for such a situation.

One can arrange equation (11) into the form

$$1/\beta = x - K/(V_{mc} - v_c). \tag{17}$$

With the values of K and V_{mc} that were obtained from the plot of equation (16) and any corresponding pair of values of x

and v_c one can obtain the value of $1/\beta$, and hence β, from equation (17).

Unfortunately it will be found in practice that the method is somewhat more sensitive to graphical construction errors than is desirable and for this reason the non-linear regression method is recommended as a preferable method for the computation of the three characterizing parameters. One useful feature of this plot is that equation (16) predicts a straight line if the system is in accord with the model presented, hence curvature of the plot is indicative of discord between the model and reality.

C. Evaluation of the parameters V_{mc}, K, *and* $1/\beta$ *by means of the non-linear regression method.* One manner of approaching the problem is to ask what values of the three parameters V_{mc}, K, and $1/\beta$ give the "best fit" of equations (10) or (11) to a set of experimental values of S and v_c. Equation (11) can be cleared of fractions to give the quadratic form

$$V_{mc}S - Sv_c - (K + V_{mc}/\beta)v_c + v_c^2/\beta = 0. \qquad (18)$$

Let

$$a = V_{mc}$$
$$b = (K + V_{mc}/\beta)$$
$$c = 1/\beta$$

then equation (18) becomes

$$aS - bv_c + cv_c^2 - Sv_c = 0. \qquad (19)$$

One wishes to ascertain those values of a, b, and c for a set of experimental values S_k of S and v_{ck} of v_c such that (19) is satisfied as closely as possible. From the form of (19) it is apparent that the condition v_c equals zero when S is zero will automatically be satisfied for any values of a, b, and c because of the lack of a constant term in equation (19). The "best fit" criterion [4] that will be used here is that

$$Q = \sum_k (a S_k - b v_{ck} + c v_{ck}^2 - S_k v_{ck})^2 \qquad (20)$$

shall be a minimum.

[4] See Appendix for discussion of "Least Squares" criterion of best fit.

One can impose the three minimizing conditions

$$\frac{\partial Q}{\partial a} = 0 \qquad \frac{\partial Q}{\partial b} = 0 \qquad \frac{\partial Q}{\partial c} = 0$$

which when applied to equation (20) give the three equations

$$\begin{aligned}
\sum_k (a S_k - b v_{ck} + c v_{ck}^2 - S_k v_{ck}) S_k &= 0 \\
\sum_k (a S_k - b v_{ck} + c v_{ck}^2 - S_k v_{ck}) v_{ck} &= 0 \qquad (21) \\
\sum_k (a S_k - b v_{ck} + c v_{ck}^2 - S_k v_{ck}) v_{ck}^2 &= 0
\end{aligned}$$

Equations (19) can be rewritten in the more convenient form

$$\begin{aligned}
a \sum_k S_k^2 - b \sum_k v_{ck} S_k + c \sum_k v_{ck}^2 S_k &= \sum_k S_k^2 v_{ck} \\
a \sum_k S_k v_{ck} - b \sum_k v_{ck}^2 + c \sum_k v_{ck}^3 &= \sum_k S_k v_{ck}^2 \qquad (22) \\
a \sum_k S_k v_{ck}^2 - b \sum_k v_{ck}^3 + c \sum_k v_{ck}^4 &= \sum_k S_k v_{ck}^3
\end{aligned}$$

The dummy subscripts, k, can be discarded as redundant since the summations are clearly indicated. It will simply be understood that the summations are to be carried out in the same manner as when the subscripts were present. With this understanding the solutions to the three equations (22) in the unknowns a, b, and c, can be written in determinant notation as

$$a = |A|/|D| \qquad b = |B|/|D| \qquad c = |C|/|D| \qquad (23)$$

$$|A| = \begin{vmatrix} \Sigma S^2 v_c & -\Sigma v_c S & \Sigma v_c^2 S \\ \Sigma S v_c^2 & -\Sigma v_c^2 & \Sigma v_c^3 \\ \Sigma S v_c^3 & -\Sigma v_c^3 & \Sigma v_c^4 \end{vmatrix} \qquad (24)$$

$$|D| = \begin{vmatrix} \Sigma S^2 & -\Sigma v_c S & \Sigma v_c^2 S \\ \Sigma S v_c & -\Sigma v_c^2 & \Sigma v_c^3 \\ \Sigma S v_c^2 & -\Sigma v_c^3 & \Sigma v_c^4 \end{vmatrix} \qquad (25)$$

$$|B| = \begin{vmatrix} \Sigma S^2 & \Sigma S^2 v_c & \Sigma v_c^2 S \\ \Sigma S v_c & \Sigma v_c^2 S & \Sigma v_c^3 \\ \Sigma v_c^2 S & \Sigma v_c^3 S & \Sigma v_c^4 \end{vmatrix} \qquad (26)$$

$$|C| = \begin{vmatrix} \Sigma S^2 & -\Sigma v_c S & \Sigma S^2 v_c \\ \Sigma S v_c & -\Sigma v_c^2 & \Sigma v_c^2 S \\ \Sigma v_c^2 S & -\Sigma v_c^3 & \Sigma v_c^3 S \end{vmatrix} \qquad (27)$$

From the manner in which, a, b, and c were previously defined one has then finally that

$$V_{mc} = a \qquad 1/\beta = c \qquad K = b - ac \qquad (28)$$

D. The interpretation of the Lineweaver and Burk plot in the case of kinetic data on intact cells. If the catalysis of a

substrate conversion is such that the rate of dependence on the concentration is of the form of equation (7) then the Lineweaver and Burk ('34) plot of 1/v against 1/S yields a straight line of slope K/V_m and intercept $1/V_m$.

Since the construction of such plots has become routine practice in the analysis of enzymatic reaction data it will be profitable to examine their meaning when the enzyme system is enclosed in an envelope that presents a diffusion barrier to its substrate. For the description of the concentration dependence of the rate of diffusion across the barrier and the rate of catalysis by the enzyme we will as before assume that equations (9) and (8) respectively are adequate. Elimination of S_i from these two equations gave equation (10).

Equation (10) can be rearranged to give

$$1/v_c = 1/V_{mc} + K/V_{mc}(1 - v_c/S \cdot \beta)S \tag{29}$$

For $S \rightarrow$ large, i.e. $1/S \rightarrow$ small, one knows that v_c cannot exceed V_{mc} and therefore can readily see that $v_c/S \rightarrow$ small. Thus

$$\lim_{S \rightarrow \infty} (v_c/S) = 0 \tag{30}$$

From (29) and (30) it is apparent that, as $1/S \rightarrow$ small, the form for $1/v_c$ approaches the form

$$[1/v_c = 1/V_{mc} + K/V_{mc}S]_{1/S \rightarrow \text{small}} \tag{31}$$

The result shown in equation (31) was also obtained by Lineweaver and Burk ('34). For small v_c and S, i.e. large $1/v_c$ and $1/S$,

$$v_c = (S - v_c/\beta)V_{mc}/K$$

or

$$\left[v_c = \frac{SV_{mc}/K}{1 + V_{mc}/K\beta} \right]_{S \rightarrow \text{small}}$$

and

$$\lim_{\substack{S \rightarrow 0 \\ v_c \rightarrow 0}} (v_c/S) = \frac{V_{mc}/K}{1 + V_{mc}/\beta K} \tag{32}$$

so that substitution of (32) into (29) gives

$$[1/v_c = 1/V_{mc} + (K/V_{mc} + 1/\beta) \cdot 1/S]_{1/S \rightarrow \text{large}} \tag{33}$$

Lineweaver and Burk and the present writer had independently verified that the slope of the limiting form (33) is $(K/V_{mc} + 1/\beta)$ but to Hearon [5] (personal communication) is due the credit for demonstrating that the ordinate intercept of the asymptote of $1/v_c$ for $1/S \rightarrow$ large is just $1/V_{mc}$.

From equations (31) and (33) one can see the shape of the $1/v_c$ against $1/S$ plot. This is given in figure 1.

Lineweaver and Burk apparently did not recognize that the ordinate intercept of the limiting form (33) is $1/V_{mc}$ for all of their curves in such cases are drawn with a positive curvature for all values of $1/S$. In a cursory examination of their data, however, the present writer sees no compelling reason why a curve of the form shown in figure 1 would not give as good a fit as those chosen by Lineweaver and Burk. Because of the nature of their interpretive methods this error does not effect the values of the parameters estimated by them.

Although these authors give three methods for the estimation of β, K, and V_{mc}, in the present writer's opinion, only one of them is trustworthy for general application. One of these methods utilizes the fact that $K/V_{mc} + 1/\beta = K'/V_{mc}$, where K' is the apparent Michaelis constant and K'/V_{mc} would be the limiting slope for $1/S \rightarrow$ large. This they arrange to obtain $K + V_{mc}/\beta = K'$. They then point out that a plot of V_{mc} against K' should give a straight line of slope $1/\beta$ and intercept K. In general the present writer sees no offhand reason to assume that an agent used to vary V_{mc} would not also vary K and β and must consequently feel that the method would be unreliable for any general application. In another method they make use of the fact that the values of the substrate concentration, $S_{\frac{1}{2}}$, giving a rate that is $\frac{1}{2}V_{mc}$ is [6]

$$S_{\frac{1}{2}} = K + V_{mc}/2\beta.$$

A plot of $S_{\frac{1}{2}}$ against V_{mc} is then supposed to yield a straight line of slope $1/2\beta$ and intercept K. This again carries the

[5] Dr. John Z. Hearon, Mathematics Panel, Oak Ridge National Laboratory.

[6] The present author has taken the liberty of correcting what must be an error in the Lineweaver and Burk discussion. They obtain that $S_{\frac{1}{2}} V_m/2 = K + V_m/2\beta$. This cannot be correct.

supposition that V_{mc} can be varied independently of K and β and must be considered risky unless one has reasonable evidence that it is so. The third method, which seems theoretically sound enough is to utilize the difference in the slopes of the asymptotes U and L of figure 1. Thus

$$\text{Slope U} - \text{Slope L} = 1/\beta.$$

This method has the drawback that any accurate graphical estimation of the slope of L is usually a difficult matter.

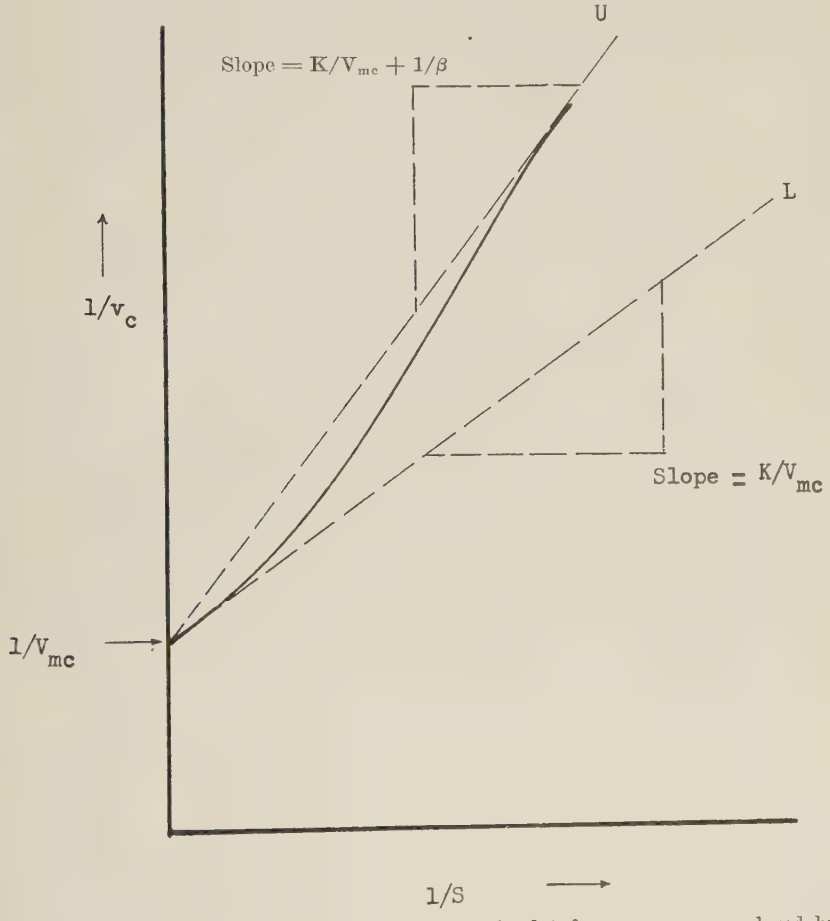

Fig. 1 The form of the Lineweaver and Burk plot for an enzyme enclosed by a membrane.

E. The results of some experiments on invertase and hexokinase as cellular components of baker's yeast. In the companion paper (Best, '55) three experiments on invertase and hexokinase as cellular components of baker's yeast are described. The rates of sucrose hydrolysis by suspensions of intact cells as a function of sucrose concentration were measured at 20°C. and 30.5°C. Rates of glucose uptake by suspensions of intact cells as a function of glucose concentration were measured at 31°C. The parameter values obtained from these three sets of data, using the non-linear regression method described earlier in the present paper, are:

1. For the sucrose-invertase system at 30.5°C.
   $V_{mc} = 5.4 \times 10^{-13}$ mols/cell min
   $1/\beta = 5.7 \times 10^{10}$ cell min/liter

2. For the sucrose-invertase system at 20°C.
   $V_{mc} = 2.1 \times 10^{-13}$ mols/cell min
   $1/\beta = 1.8 \times 10^{11}$ cell min/liter
   $K = 1.0 \times 10^{-3}$ mols/liter

3. For the glucose-hexokinase system at 31°C.
   $V_{mc} = 1.1 \times 10^{-15}$ mols/cell min
   $1/\beta = 1.1 \times 10^{12}$ cell min/liter
   $K = 6.4 \times 10^{-4}$ mols/liter

From the temperature dependence of β for sucrose a Q_{10} of 3.0 was computed. In the companion paper these values were compared with some of the permeability values obtained by other investigators (Jacobs and Stewart, '32; Landahl, '39; Rashevsky, '48) and found to fall well within the range of values reported for other substances and cells. The interpretation of these values in terms of the cytochemical structure of the yeast cell was discussed at some length.

It will be profitable to utilize these values to discuss some of the assumptions made previously in this paper in the derivation of the model.

F. Assumption of the steady state with regard to entry and conversion. It was assumed, under the conditions of these experiments, that the rate of entry is equal to the rate of catalytic conversion, i.e. that the steady state condition as-

sumed in the derivation presented in section A is satisfied. Inasmuch as the external substrate concentration was steadily changing and the rates were obtained by measuring the slope of the S versus t curve at zero time, one might ask whether the steady or quasi-steady state is really approximated under the conditions of the experiments. One can give an approximate [7] treatment that will serve to set the limits on the error incurred by possible failure of the steady state assumption.

Any rate behavior demonstrated by a Michaelis and Menten system will lie between the limits set by the behavior of a first order process and a constant rate process. This being the case, it is clear that if one can establish that the steady state condition is effectively satisfied in each of these limiting cases, then it must also be satisfied by the Michaelis and Menten system.

The concentration of substrate in the medium can, for not too large values of t, be represented by the expression

$$S = S_o - Rt \tag{34}$$

where S_o is the initial concentration and t is the time. R is assumed to be constant (independent of t) and will be the rate of decrease of the concentration in the medium in the neighborhood of t equal to zero.

If V is the cell volume and k the rate constant per cell (where the reaction in the cell is assumed to behave first order with respect to the internal concentration) then

$$V\, dS_i/dt = \beta \cdot (S - S_i) - kS_i \tag{35}$$

The parameter has the meaning attached to it previously. One can rearrange (35) and introduce (34) to obtain

$$dS_i/dt + (k + \beta)S_i/V = \beta S_o/V - \beta Rt/V. \tag{36}$$

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[7] For a discussion of the assumptions involved in the use of the differential equation

$$dS_i/dt = Q + \frac{\beta}{V}(S - S_i),$$

in which Q and S_i are the spatial means of the reaction rate and internal concentration, to replace the equation of continuity and its associated boundary conditions when S is constant in time, see N. Rashevsky (Chap. I, '48) and John Z. Hearon ('53, I and II).

Equation (36) can be readily integrated to

$$S_i = \frac{\beta}{(k+\beta)}\left(S_o + \frac{RV}{(k+\beta)}\right)\left(1 - e^{\frac{-(k+\beta)t}{V}}\right) - \frac{\beta R t}{(k+\beta)} \quad (37)$$

From the data obtained in the glucose experiment (Best, '55)

$$\begin{aligned} \beta &= 10^{-9} \text{ cc./cell. min.} \\ k &= V_{mc}/K = 1.7 \times 10^{-9} \text{ cc./cell min.} \\ V &= 2.2 \times 10^{-11} \text{ cc.} \\ R &= 20 \times 10^{-9} \text{ mols/cc. min.} \\ S_o &= 12 \times 10^{-7} \text{ mols/cc.} \end{aligned} \quad (38)$$

One can now ask how soon the exponential in the transient term

$$1 - e^{\frac{-(k+\beta)t}{V}}$$

of equation (37) will decay to its 1/e value. In the case of the glucose experiments this will occur when

$$t = V/(k+\beta) = .0082 \text{ min.} = 0.5 \text{ seconds}$$

One can ask further whether the term $RV/(k+\beta)$ is sufficiently small in comparison to S_o that it could be safely neglected. For the glucose experiments

$$RV/(k+\beta) \cong 16 \times 10^{-11} \text{ mols/cc.} << 12 \times 10^{-7} \text{ mols/cc.} = S_o$$

On the basis of these order of magnitude calculations equation (37) can be simplified to the form

$$S_i = \frac{\beta}{(k+\beta)}(S_o - Rt) \quad (39)$$

If one put such a cell in a medium of constant substrate concentration S and allowed it to attain a steady state then the relationship between the internal and external substrate concentrations would be just

$$S_i = \frac{\beta}{(k+\beta)} S \quad (40)$$

From equations (34) and (39) it is apparent that the cells, under the experimental conditions in question, maintain the relation (40) in spite of the fact that S is changing with time. One could therefore conclude that such a cell, under the conditions of the glucose experiment previously mentioned, would

exhibit essentially steady state behavior at any time after the first 1.5 seconds.

In the case of a cell with constant rate of consumption per cell, q, one can replace equation (35) with

$$V\, dS_i/dt = \beta \cdot (S - S_i) - q. \qquad (41)$$

Introducing (34) into (41) and integrating gives

$$S_i = (S_o - q/\beta + RV/\beta)(1 - e^{-\beta t/V}) - Rt \qquad (42)$$

In choosing the value of the constant q to be inserted into equation (42) some care must be exercised. A moment's reflection will show that if a value of q greater than βS_o is chosen, then the physical absurdity of a negative steady state value for S_i would result when S is maintained constant a S_o. If S_i is zero at t equal to zero and S were held constant at S_o, then S_i would increase monotonically to its steady state value. By choosing q equal to the experimental value of v_c that would correspond to S_o, one obtains a value of $S_o - q/\beta$ that is as small as would be encountered for a particular value of S_o yet at the same time avoiding the negative S_i pitfall. It is important that the value of $S_o - q/\beta$ considered be as small as the value which it physically attains since one wishes to show in equation (42) that the condition

$$S_o - q/\beta >> RV/\beta$$

is satisfied for the experiments under discussion.

For the glucose uptake experiments one finds from figure 3 of the companion paper (Best, '55) that the value of $S_o = 1.2 \times 10^{-6}$ mols/cc corresponds to a $v_c = 5.3 \times 10^{-16}$ mols/cell min. From the values of β, R, and V given in (38) one obtains that

$$RV/\beta \cong 4.4 \times 10^{-10} \text{ mols/cc}$$

and taking $q = v_c$ (experimental for S_o) one has

$$q/\beta \cong 5 \times 10^{-7} \text{ mols/cc.}$$

Hence

$$S_o - q/\beta \cong 7 \times 10^{-7} \text{ mols/cc} >> 4.4 \times 10^{-10} \text{ mols/cc} \cong RV/\beta.$$

The term $e^{-\beta t/V}$ of equation (42) will drop to a value of 1/e when

$$t = V/\beta = 1.3 \text{ seconds.}$$

On the basis of these considerations of orders of magnitude, equation (42) can be written, without appreciable error, after about the first 4 seconds, as

$$S_i = S_o - q/\beta - Rt \tag{43}$$

or, by recalling equation (34), as

$$S_i = S - q/\beta \tag{44}$$

Equation (44) is, however, just the relation between S and S_i that would be obtained if the cell were in a medium of constant concentration S and had attained a steady state. From the argument given above with regard to the transient terms in the cases where the rate is linear and constant respectively with regard to S_i one can conclude that the yeast cells in the experiments involving glucose have effectively attained the steady state relation between S and S_i in from 1.5 to 4 seconds.

Using equations (37) and (42) one can now proceed to carry out a similar set of considerations for the invertase experiments. It will be sufficient in this case to merely carry out the calculation for the 20°C. case since the variation of the parameters with a 10°C. temperature rise will not be sufficient to invalidate the order of magnitude conclusions obtained.

One has

$$\begin{aligned} \beta &= 5.6 \times 10^{-9} \text{ cc/cell min} \\ K &= 10^{-6} \text{ mols/cc} \\ V_{mc} &= 2.1 \times 10^{-13} \text{ mols/cell min} \\ V &= 2.2 \times 10^{-11} \text{ cc.} \\ R &= 20 \times 10^{-8} \text{ mols/cc. min} \\ S_o &= 3 \times 10^{-5} \text{ mols/cc.} \end{aligned} \tag{45}$$

Then for the case of linear rate dependence upon S_i one can, as before, take $k = V_{mc}/K = 2.1 \times 10^{-7}$ cc/cell min.

and since $k >> \beta$ we can consider

$$k + \beta = 2.1 \times 10^{-7} \text{ cc/cell min.}$$

In the case of the data regarding sucrose hydrolysis given by (45), the term $e^{-\frac{(k+\beta)}{V}t}$ of equation (37) will decay to a value of 1/e when $t = V/(k + \beta) = 10^{-4}$ min. = .02 seconds.

Then in about .06 seconds the exponential term will have decayed to less than 1/10 and can be neglected for practical purposes.

One also finds that

$$RV/(k+\beta) \cong 2 \times 10^{-11} \text{ mols/cc} << 3 \times 10^{-5} \text{ mols/cc} = S_o$$

so that equation (37) reduces to the steady state relation between S and S_i given by (40).

In an investigation of equation (42) for the data given in (45) one finds from figure 1 of the companion paper a value of

$$q = v_c = 15 \times 10^{-14} \text{ mols/cell min.}$$

corresponding to S_o. Thus $q/\beta \cong 2.7 \times 10^{-5}$ mols/cc, so

$$S_o - q/\beta = 3 \times 10^{-6} \text{ mols/cc} >> 8 \times 10^{-10} \text{ mols/cc} = RV/\beta.$$

The exponential term $e^{-\beta t/V}$ of equation (42) will decay, in this case, to tis 1/e value when

$$t = V/\beta \cong 0.5 \times 10^{-2} \text{ min or } 0.3 \text{ seconds.}$$

Hence, after about one second the relationship between S and S_i can, for all practical purposes, be reduced to the steady state relation given by equation (44).

G. Assumption of no concentration gradients. In the present treatment it was assumed that no appreciable gradients of the substrate were present in the interior of the cell. This assumption, along with the assumption of complete mixing of the external medium, implies that the coefficient β is just Ah, where A is the surface area of the cell and h is the membrane permeability. In order to examine this assumption more closely it will be instructive to discuss the treatment presented in this paper in the light of those given by Rashevsky ('48, Chap. II), and Hearon ('53, I and II).

Rashevsky ('48, p. 20) introduced a quantity Λ, having the dimensions of reciprocal time, which he called the "diffusion resistance." The meaning of Λ is most clearly demonstrated by the manner in which it enters into the conservation equation

$$dC/dt = Q - (C - C_o)/\Lambda \qquad (46)$$

where C is the spatial average of the concentration of a substance in a cell at any particular time t, C_o the concentration

in the external medium at an infinite distance away from the cell, and $\bar{Q}$ the spatial average of Q, the rate of production of the substance per unit volume of the cell. The "approximation method" set forth by Rashevsky (Chap. I and II, '48) in his final result asserts that equation (46) is true and that factors such as cell shape, size, internal enzyme distribution, etc. can be incorporated into the parameter Λ. As Hearon ('53, I) shows this is equivalent to the assertion that

$$(1/V)\int_A \vec{J}\cdot d\vec{\sigma} = (\bar{C} - C_o)/A \qquad (47)$$

in which J is the flux vector through the element of area $d\vec{\sigma}$, V is the cell volume, and the integral is taken over the cell surface. The imposition of a steady state is thus equivalent to setting

$$d\bar{C}/dt = 0 \qquad (48)$$

from which one has that

$$\bar{Q} = (\bar{C} - C_o)/\Lambda. \qquad (49)$$

Rashevsky ('48, Chap. II) has shown that if Q is constant (independent of the concentration) in a spherically symmetric cell, then

$$\Lambda = r_o/3h + r_o^2/3D_e + r_o^2/15D_i \qquad (50)$$

where r_o is the cell radius, D_e the diffusion coefficient of the substance in the external medium, D_i its diffusion coefficient inside the cell, and h the permeability of the cell membrane for the substance.

If one multiplies equation (49) through by the volume, V, of the cell, then one obtains that

$$V\bar{Q} = (\bar{C} - C_o)V/\Lambda \qquad (51)$$

and since $v_c = -V\bar{Q}$, then

$$v_c = (C_o - \bar{C})V/\Lambda. \qquad (52)$$

From a comparison of equations (9) and (52) it is apparent that

$$\beta = V/\Lambda. \qquad (53)$$

Hearon has demonstrated that if Q is *not* concentration independent and internal concentration gradients *not* negligible

then for a spherical cell one must add a correction ε to the value of Λ given by equation (50). He has shown ('53, I) that

$$\Lambda = \Lambda_o + \epsilon \qquad (54)$$

where Λ_o is the value of Λ for a spherical cell of constant Q given by equation (50) and

$$\epsilon = (r_o^2/10D_1)(1 - \bar{r^2}Q/\bar{r^2}\,\bar{Q}) \qquad (55)$$

in which the bar indicates the spatial average of the quantity over the cell volume.

Equation (7) along with equations (53), (54), (55) can be used to answer two important questions regarding the present work. First, is it permissible to ignore the internal and external gradients and consider only the resistance to entry presented by a relatively thin shell or membrane like barrier? Second, can one account for the diffusion resistance encountered by either of the substrates investigated in the companion paper in passing from the external medium to the cellular location of its respective enzyme on the basis of incomplete mixing or stagnant water layers surrounding the cell.

It will be assumed for the purposes of argument that the experimental values obtained in the companion paper represent at least the correct orders of magnitude for the quantities they are supposed to represent.

Consider the two limiting cases of a substance whose rate of disappearance follows the Michaelis and Menten rate expression. If the substrate concentration is small, i.e. very much less than K, then its rate of disappearance, Q, will be proportional to its concentration with V_m/K appearing as the constant of proportionality. If the substrate concentration is large, i.e. very much greater than K, then $Q = V_m$ and will be independent of the substrate concentration. If one can demonstrate the absence of any contribution to β by internal or external concentration gradients in each of these limiting situations it seems clear that there will be no such contribution for any intermediate values of substrate concentration. This is the path of argument that will be followed.

In the case that Q is constant for a spherical cell one can write, from equations (50), (53), and the formulas for the area and volume of a sphere, that

$$1/\beta = (1/Ah)(1 + r_o h/D_e + r_o h/5D_i) \tag{56}$$

or that

$$1/\beta - (r_o/A)(1/D_e + 1/5D_i) = 1/Ah. \tag{57}$$

From (57) it becomes apparent that the assertion

$$\beta \cong Ah \tag{58}$$

for such a cell is equivalent to the assertion

$$(1/D_e + 1/5D_i) r_o/A << 1/\beta. \tag{59}$$

Hearon ('53, II) has investigated the correction term ϵ of equations ('54), and (55) for the case of a spherically symmetric cell in which $Q = -kC$. The concentration C of the substance being consumed will, in general, be a function of the distance r from the center of the cell. He has shown that

$$\epsilon = \frac{r_o^2}{15\,D_i}\,\xi\,(x) \tag{60}$$

where

$$x = r_o \sqrt{k/D_i}$$

and has computed and given in tabular form the values of $\xi(x)$ corresponding to various values of x.

From equations (50), (53), (54), and (60) one can write that

$$1/\beta - (r_o/A)(1/D_e + 1/5D_i + (1/5D_i)\xi(x)) = 1/Ah \tag{61}$$

The assertion made by equation (58) is equivalent, therefore, to the assertion that

$$(1/D_e + 1/5D_i + \xi(x) \cdot 1/5D_i)\, r_o/A << 1/\beta \tag{62}$$

for the case of a cell in which the rate of catalysis is proportional to the substrate concentration at the site of the catalyst.

Since $k = V_m/K$, its value in terms of the parameters of the present discussion will be

$$k = V_{mc}/VK$$

and

$$x = r_o \sqrt{V_{mc}/D_i KV} \tag{63}$$

The effect of very rapid mixing of the external medium is to make D_e infinite. One can therefore establish the possible

range of variation of β as a function of the degree of mechanical mixing. In the case that Q is constant

$$\underset{\text{for very rapid mechanical mixing}}{(1/Ah + r_o/5D_iA)} \leq 1/\beta \leq \underset{\text{for no mechanical mixing}}{(1/Ah + r_o/5D_iA + r_o/D_eA)} \quad (64)$$

For the case that $Q = -kC$

$$\underset{\text{for no mechanical mixing}}{(1 + r_oh/D_e + r_oh/5D_i + r_oh\,\xi(x)/5D_i)\cdot 1/Ah} \geqq 1/\beta \geqq \underset{\text{for very rapid mechanical mixing}}{(1 + r_oh/5D_i + r_oh\,\xi(x)/5D_i)\cdot 1/Ah} \quad (65)$$

It is clear from (64) and (65) that establishing the validity of the statement

$$1/\beta >> r_o/D_eA \quad (66)$$

will mean the absence of an appreciable contribution to the value of $1/\beta$ by possible incomplete mixing.

If one can demonstrate that the condition expressed by (59) is satisfied then one will also have demonstrated automatically that (66) is satisfied.

For the purposes at hand one can, from the measurements performed in the companion paper, take

$$r_o \cong 10^{-4} \text{ cm.} \quad (67)$$

The values given (Int. Critical Tables, '29) for the diffusion coefficients of glucose and sucrose in aqueous solution at 20°C. are 0.60×10^{-5} cm^2/sec and 0.42×10^{-5} cm^2/sec respectively. As a rough value for both therefore one can consider

$$D_e \cong 0.5 \times 10^{-5} \text{ cm}^2/\text{sec} = 3 \times 10^{-4} \text{ cm}^2/\text{min} \quad (68)$$

as being close enough. One does not know the exact value of the diffusion coefficient, D_i, for these sugars inside the cell, however, from observations on the diffusion of such light molecular weight non-electrolytes through gels one has no reason to believe (Glasstone, '46, page 1262) that D_i would differ very greatly from D_e. If D_i is much larger than D_e, then the actual error would simply be smaller than that calculated by assuming them equal. In order to be on the safe side in estimating the maximum error likely to be incurred let us assume that D_i is only one fifth as large as D_e. Thus one has

$$D_i \cong 10^{-6} \text{ cm}^2/\text{sec} = 6 \times 10^{-5} \text{ cm}^2/\text{min.} \quad (69)$$

From the values given by (67), (68), and (69) one finds that

$$(1/D_e + 1/5D_i)r_o/A = 5 \times 10^6 \text{ cell min/cc} << 1 \times 10^9 \text{ cell min/cc} = 1/\beta \text{ for glucose}$$

$$<< 6 \times 10^7 \text{ cell min/cc} = 1/\beta \text{ for sucrose at } 30.5°\text{C.}$$

$$<< 6 \times 10^8 \text{ cell min/cc} = 1/\beta \text{ for sucrose at } 20°\text{C.}$$

Hence the conditions expressed by (59) and (66) are fulfilled in all of the experiments.

One can now inquire whether relation (62) is satisfied in the experiments of the companion paper. Taking $V_{mc}/K = 1.7 \times 10^{-9}$ cc/cell min for the glucose-hexokinase experiment, one finds that $x = 0.1$ and from Hearon ('53, II) that $\xi(x) = -0.002$, and $r_o\xi(x)/5D_iA = -2 \times 10^3$ cc/cell min for glucose. If one uses $V_{mc}/K = 2 \times 10^{-7}$ cc/cell min for the sucrose experiments, then $x = 1$ and one finds that $\xi(x) = -0.03$ so that $r_o\xi(x)/5D_iA = -3 \times 10^4$ cc/cell min for sucrose. Thus for the experiments of the companion paper one has

$$(1/D_e + 1/5D_i + \xi(x)/5D_i)\, r_o/A = 5 \times 10^6 \text{ cell min/cc},$$

which, as already demonstrated, is very much less than any of the values of $1/\beta$ measured, so that condition (62) is adequately fulfilled.

One may conclude, therefore, from the arguments presented above that in these experiments it is permissible to ignore the internal and external concentration gradients insofar as their contributions to the value of $1/\beta$ are concerned and β has the interpretation stated by relation (58).

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APPENDIX

Some comments on the least squares criterion of best fit

Consider a set of associated pairs of the variables x and y. Each such pair will constitute a point x_k, y_k in the x, y plane. The data obtained from experiments of the type described in the companion paper (Best, '55) will yield a set of such points.

Suppose, as in the case considered in this paper, one has some hypothesis regarding the functional relation between y and x that might be expressed as

$$f(x,y) = 0 \tag{1}$$

or in the form of an explicit function

$$y = \phi\,(x). \tag{2}$$

Usually the hypothesis does not specify the exact function f(x,y) or $\phi(x)$ but only the form. This form postulated by the hypothesis usually contains one or more parameters whose values must be determined from the experimental data. Such specification of the parameter values amounts to selecting one function f or ϕ out of all those having the form prescribed by the hypothesis.

If the error incurred in fixing the value of x (which will be considered the independent variable) is considerably less than that involved in the measurement of y then the best (most statistically efficient) estimate of the parameters characterizing $\phi(x)$ will, if the residual errors, $(y_k - \phi(x_k))$, are assumed to be normally distributed with variance independent of x, be that obtained by minimizing

$$U = \sum_k (y_k - \phi\,(x_k))^2 \tag{3}$$

with respect to the parameter values. It should be noted that this procedure is not quite the same as minimizing

$$Q = \sum_k f(x_k, y_k)^2 \tag{4}$$

In the case of a linear regression form

$$y = a + bx$$

both (3) and (4) become the same. In general, however, the form proferred by a hypothesis will not give an explicit solution for y that is linear in x and therefore application of (3) and (4) will not in general yield the same parameter values.

As the sum of the squares, U, of the residual errors becomes smaller, however, Q will also become smaller and the parameter values estimated by the two criteria will approach one another. This property suggests that (4) might serve to yield reasonably good approximations of the parameters when the application of (3) is not feasible.

Consider the form

$$av_c^2 - bv_c + cS - Sv_c = 0$$

obtained from the reaction model discussed in this paper. The explicit solution for v_c that is consistent with reality is

$$v_c = \frac{(b + S) - \sqrt{(b + S)^2 - 4acS}}{2a}.$$

Then according to (3)

$$U = \sum_k \left[v_{ck} - \frac{(b + S_k) - \sqrt{(b + S_k)^2 - 4acS_k}}{2a} \right]^2$$

Imposition of the conditions

$$\partial U/\partial a = \partial U/\partial b = \partial U/\partial c = 0$$

yields a set of equations that are neither linear nor homogeneous in the parameters a, b, and c. The solution of these equations for the parameter values, while perhaps not hopeless, was not apparent to the present writer and is not encompassed by the theory of determinants. In order to circumvent this difficulty the approach provided by the minimization of Q was utilized. This method, as has already been demonstrated in the body of the paper, does lead to a set of equations that are linear in the parameters a, b, and c from which one can obtain solutions by straightforward application of the theory of determinants. In the present case the residual errors of v_c (for the data presented in the companion paper) were sufficiently small that the parameter values so obtained probably lie fairly close to the most efficient estimates of their values.

LITERATURE CITED

BEST, JAY BOYD 1955 The inference of intracellular enzymatic properties. II. J. Cell. and Comp. Physiol., *46:* 29.

BRIGGS, G. E., AND J. B. S. HALDANE 1925 A note on the kinetics of enzyme action. Biochem. J., *19:* 338.

GLASSTONE, S. 1946 Textbook of physical Chemistry, 2nd. ed., New York: D. Van Nostrand Company, Inc.

HALDANE, J. B. S. 1930 Enzymes, New York: Longmans Green and Co.

HEARON, JOHN Z. 1949a The steady state kinetics of some biological systems. I. Bull. Math. Biophysics, *11:* 29–49.

———— 1949b The steady state kinetics of some biological systems. II. Bull. Math. Biophysics, *11:* 83.

———— 1952 Rate behavior of metabolic systems. Physiological Rev., *32:* 499–523.

———— 1953 Comments on the approximate solution of the diffusion equation. I. Bull. Math. Biophysics, *15:* 21–29.

———— 1953 Comments on the approximate solution of the diffusion equation. II. Bull. Math. Biophysics, *15:* 111–119.

JACOBS, M. H., AND D. R. STEWART 1932 Permeability of marine eggs. J. Cell. and Comp. Physiol., *1:* 71.

LANDAHL, H. D. 1939 Mathematical biophysics of cell respiration. II. Bull. Math. Biophysics, *1:* 1–17.

———— 1948 Mathematical Biophysics, by N. Rashevsky, rev. ed. Chap. V. University of Chicago Press.

LINEWEAVER, H., AND D. BURK 1934 J. Amer. Chem. Soc., *56:* 658.

MICHAELIS, L., AND M. L. MENTEN 1913 Die Kinetik der Invertinwirkung. Biochemische Zeitschrift, *49:* 333.

RASHEVSKY, N. 1948 Mathematical Biophysics, rev. ed., Chicago: University of Chicago Press.

WEICHHERZ, J. 1929 Die Kinetik der Zellreaktionen. I. Z. Physik. Chem. A., *145:* 330.

———— 1931 Die Kinetik der Zellreaktionen. II. Biochemische Zeitschrift, *238:* 325.

THE INFERENCE OF INTRACELLULAR ENZYMATIC PROPERTIES FROM KINETIC DATA OBTAINED ON LIVING CELLS

II. A STUDY OF HEXOKINASE AND INVERTASE AS CELLULAR COMPONENTS OF BAKER'S YEAST

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THREE FIGURES

It is the purpose of this paper and a companion one (Best, '55) to demonstrate a method by means of which some of the properties of enzyme in living cells can be investigated and the membrane permeability of the cells to their substrates measured.

Different methods for the measurement of the permeability constant (Collander and Barlund, '33; Ruhland and Hoffman, '25; Jacobs and Stewart, '32; Jacobs, '30, '31, '50; Parpart, '35) have been used to investigate the structure of the cell membrane. More scarce is information concerning values of the permeability constant for substances metabolically utilized and converted by the cell in question. The reason lies in the limitations imposed by the methods hitherto available for the technical execution of such measurements. These limitations are made clearer by a brief examination of the methods of permeability measurement.

The most obvious and direct method is that of sampling and direct analysis of the cellular contents at various times.

[1] This work was done in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology of the University of Chicago. The writer was a Public Health Service fellow of the National Microbiological Institute at the time.

Thus, from a knowledge of the external and internal concentrations of the substance in question, one can directly compute the permeability of the membrane to the test substance. Direct tapping of the cellular contents, without centrifugation from the medium and disruption of the cell, is possible only for very large plant cells such as that of *Chara ceratophylla* used by Collander and Barlund ('33). Application of such direct procedures to cells of more conventional size necessitates that the test substance be consumed or produced only to a negligible extent during the course of the experiment and the rate of entry be slow in comparison to the time required to sample the cellular contents.

Practically all other methods depend upon osmotic pressure changes between the inside and outside of the cell brought about by the entry or departure of the test substance. From the standpoint of yielding quantitative results and applicability to a wide range of cell sizes, probably the best methods are those which depend upon the theoretical interpretation, derived by Jacobs and Stewart ('32), of the transient volume changes induced in the cell by the test substance. The considerations upon which permeability measurements of this type are based likewise include the assumption that the test substance is, for practical purposes, conserved during the course of the experiment. These methods then preclude their use for the measurement of the permeability for test substances that are metabolized at any appreciable rate by the cell under investigation.

To measure the membrane permeability of a cell to a substance that undergoes metabolic conversion at a rate appreciable in comparison to the rate of entry through the membrane the rate of conversion must be taken into consideration in an adequate manner. Although a more thorough presentation of the mathematical aspects of the problem is reserved for the companion paper (Best, '55), some remarks are necessary for the present discussion.

Consider the situation presented by a suspension of cells in a test medium. For a substance entering the cells of the sus-

pension the material balance, under steady state conditions, will be just

$$v_c = \beta \cdot (S - S_i) \quad (1)$$

$$v_c = Q(S_i), \quad (2)$$

where S is the concentration of substrate in the external medium, S_i its concentration in the cell, v_c the rate of entry of the substance, and $Q(S_i)$ the rate at which it is consumed (catalytically converted) per cell. $Q(S_i)$ will be a function of S_i. In order to measure the value of β (which under certain circumstances will turn out to be just the permeability constant, h, of the membrane multiplied by the surface area of the cell) one must have actual knowledge of the value of S_i or eliminate it from equations (1) and (2).

If one knew not just the form but the actual function $Q(S_i)$ then for any steady state situation one would need to know only the value of v_c and S in order to know S_i and hence, from equation (1), β. In the converse situation, if, v_c, and S were known then the value of S_i corresponding to a particular value of Q could be calculated. Thus, if β were known, one could experimentally determine $Q(S_i)$. Unfortunately neither β nor $Q(S_i)$ are known. One must conclude that, for a substrate, the measurements of β and the formal kinetic properties of the intracellular enzyme, *in situ,* are inextricably bound together and must be carried out simultaneously.

In the companion paper a theoretical model is set up in which the functional form of $Q(S_i)$ is assumed to be that of the Michaelis and Menten ('13) rate expression for enzymatically catalyzed processes. Three possible procedures for application of this model to the analysis of experimental data are discussed. One of these, the non-linear regression method, is much the preferable and will be used for the analysis of the data presented in the present paper.

THEORY

1. The Michaelis and Menten enzyme enclosed by a membrane. If one assumes that the rate of entry of a substrate into the cell is adequately given by equation (1) and the rate

of catalysis inside the cell is given by the rectangular hyperbolic relation

$$v_c = V_{mc}S_i/(S_i + K) \tag{3}$$

of the Michaelis and Menten ('13) formulation, then it can be shown (Best, '55) that

$$V_{mc} - v_c = K/(S/v_c - 1/\beta) \tag{4}$$

Equation (4) does not contain the quantity S_i which is technically difficult to measure.

2. Evaluation of V_{mc}, K, *and* $1/\beta$ *by the non-linear regression method.* Equation (4) can be written in the form

$$a\,S - b\,v_c + c\,v_c^2 - S\,v_c = 0 \tag{5}$$

where $V_{mc} = a$, $K = b - ac$, $1/\beta = c$. One can ask those values of a, b, and c for a given set of experimental values S_k of S and v_{ck} of v_c such that equation (5) is satisfied as closely as possible. The "best estimate" values of a, b, and c and, from them, K, V_{mc}, and $1/\beta$, have been shown (Best, '55) to be $a = |A|/|D|$, $b = |B|/|D|$, $c = |C|/|D|$, where $|A|$, $|B|$, $|C|$, and $|D|$ are the values of the determinants

$$|A| = \begin{vmatrix} \Sigma S^2 v_c & -\Sigma v_c S & \Sigma v_c^2 S \\ \Sigma S v_c^2 & -\Sigma v_c^2 & \Sigma v_c^3 \\ \Sigma S v_c^3 & -\Sigma v_c^3 & \Sigma v_c^4 \end{vmatrix} \tag{6}$$

$$|D| = \begin{vmatrix} \Sigma S^2 & -\Sigma v_c S & \Sigma v_c^2 S \\ \Sigma S v_c & -\Sigma v_c^2 & \Sigma v_c^3 \\ \Sigma v_c^2 S & -\Sigma v_c^3 & \Sigma v_c^4 \end{vmatrix} \tag{7}$$

$$|B| = \begin{vmatrix} \Sigma S^2 & \Sigma S^2 v_c & \Sigma v_c^2 S \\ \Sigma S v_c & \Sigma v_c^2 S & \Sigma v_c^3 \\ \Sigma v_c^2 S & \Sigma v_c^3 S & \Sigma v_c^4 \end{vmatrix} \tag{8}$$

$$|C| = \begin{vmatrix} \Sigma S^2 & -\Sigma v_c S & \Sigma S^2 v_c \\ \Sigma S v_c & -\Sigma v_c^2 & \Sigma v_c^2 S \\ \Sigma v_c^2 S & -\Sigma v_c^3 & \Sigma v_c^3 S \end{vmatrix} \tag{9}$$

METHODS

A series of flasks on a shaker in a constant temperature bath were prepared, each with a given volume of sugar (sucrose or glucose) solution of known concentration in phosphate buffer. At zero time each flask was inoculated with a given volume of a suspension of washed baker's yeast of known cell population density. The phosphate buffer used for washing and suspend-

ing the cells was 0.1 M KH_2PO_4 at pH 4.5 in the sucrose hydrolysis experiments and comprised of 0.03 M K_2HPO_4, 0.02 M KH_2PO_4, and 0.05 M NaCl at pH 7.0 in the glucose uptake experiment. The washed cells needed no added Ca or Mg for maximal rates of glucose uptake. Cell population densities were determined by optical density measurements.

At various times after inoculation aliquots were removed from each flask and each quickly delivered into a preheated test tube in a boiling water bath. The time was recorded at the moment the first fluid from the aliquot pipet entered the tube. Approximately three minutes after receiving the aliquots the tubes were removed from the boiling water bath and cooled under running tap water. The tubes were centrifuged and samples of supernatant solution withdrawn for sugar analysis.

Sucrose analyses were carried out by determining the difference in reducing sugar values before and after acid hydrolysis. For the analysis of glucose, Somogyi's ('45) $Ba(OH)_2$ and $ZnSO_4$ reagents were added to the sample in accurately measured amounts and the precipitate and cells removed by centrifugation. Samples of this supernatant solution were withdrawn for reducing sugar determination. The samples were immersed in a boiling water bath for six minutes to remove any acetaldehyde that might be present. All reducing sugar measurements were made with the ceric sulfate titration procedure of Miller and Van Slyke ('36).

DATA AND CALCULATIONS

1. Data from the sucrose hydrolysis (invertase) experiments. Data obtained from those experiments conducted to investigate the intracellular properties of invertase by measuring the rate of hydrolysis of sucrose by intact baker's yeast cells are presented in tables 1 and 2. S refers to the substrate concentration and t the time after inoculation.

The data of tables 1 and 2 were plotted in graphical form to obtain a curve of sucrose concentration as a function of time for each of the reaction vessels. The initial slope of each curve

TABLE 1

Sucrose hydrolysis by intact cells of baker's yeast at 20°C.

FLASK	A		B		C		D	
ALIQUOT	S (mg %)	t (min)	S (mg %)	t (min)	S (mg %)	t (min)	S (mg %)	t (min)
0	2000 [1]	0	1200 [1]	0	800 [1]	0	160 [1]	0
1	1780	11.5	1097	10.7	736	13.2	153	12.5
2	1495	25.5	1000	24.7	688	27.5	150	26.2
3	1271	36.7	901	35.9	679	38.5	147	37.3
4	1097	51.4	830	50.4	608	53.3	141	52.3
5	977	60.0	802	59.5			137	61.5
N	2.80 n		1.40 n		0.90 n		0.30 n	
cells per ml	$n = 1.0 \times 10^8$							

[1] Zero time concentrations were obtained by multiplying the concentration of sucrose solution prepared from a gravimetric standard by the appropriate factor of dilution.

TABLE 2

Sucrose hydrolysis by intact cells of baker's yeast at 30.5°C.

FLASK	A		B		C		D		E	
ALIQUOT	S (mg %)	t (min)	S (mg %)	t (min)	S (mg %)	t (min)	S (mg %)	t (min)	S (mg %)	t (min)
0	2500 [1]	0	2040 [1]	0	1500 [1]	0	1000 [1]	0	500 [1]	0
1	1972	12.5	1527	12.5	1085	11.4	739	11.0	321	13.1
2	1545	26.4	1079	26.0	790	24.8	521	23.9	236	23.0
3	725	46.3	572	45.9	416	44.7	284	44.0	116	43.0
4	420	62.8	277	62.4	208	61.1	152	60.1	35	59.2
N	3.12 n		2.50 n		2.16 n		1.74 n		1.74 n	
cells per ml	$n = 1.0 \times 10^8$									

[1] Zero time concentrations were obtained by multiplying the concentration of sucrose solution prepared from a gravimetric standard by the appropriate factor of dilution.

TABLE 3

FLASK	S mols/liter $\times 10^2$	v mols/ml min $\times 10^8$	v_c mols/cell min $\times 10^{14}$	TEMP.
A	5.83	58.1	20.6	20°C.
B	3.50	24.3	17.3	
C	2.34	10.4	11.5	
D	0.47	0.99	3.3	
A	7.30	125	40.0	30.5°C.
B	5.95	119	47.6	
C	4.38	106	49.0	
D	2.92	72.4	41.6	
E	1.46	40.8	23.5	

was measured and taken as the rate, v, of sucrose hydrolysis corresponding to the initial concentration of sucrose. If the rate, v, is expressed in mols/ml min and divided by the cell population density, N, (expressed in number cells/ml) then one obtains the rate per cell, v_c, expressed in mols/cell min. These are given in table 3. The set of values of S and v_c, which is contained in table 3, are plotted in graphical form in figure 1.

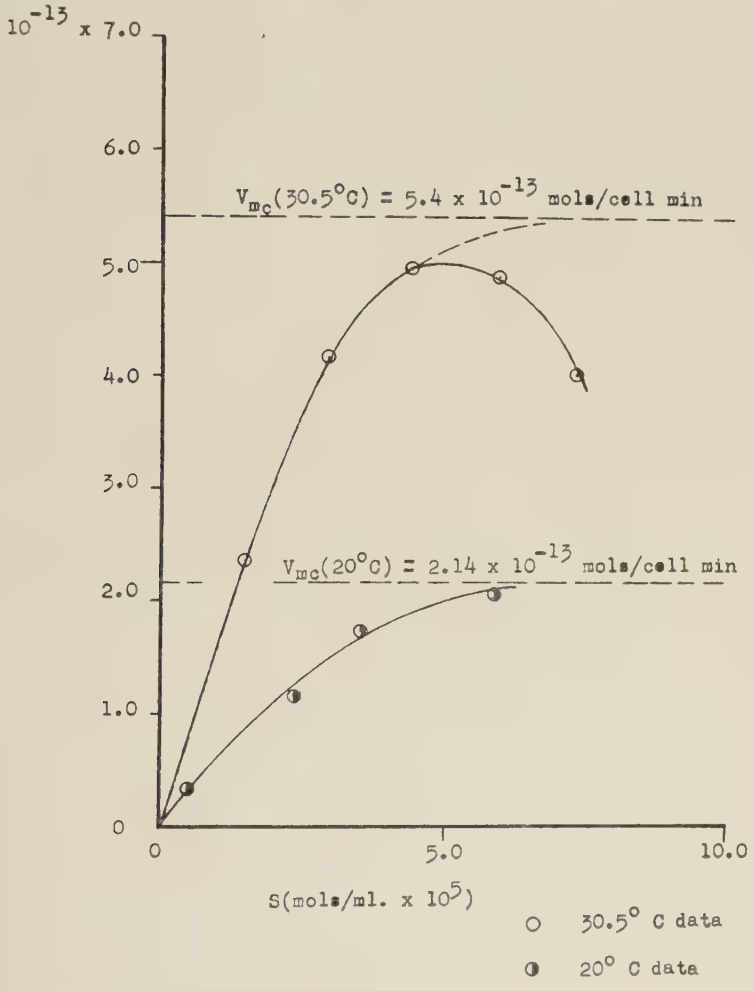

Fig. 1 Rate of sucrose hydrolysis per cell by intact baker's yeast as a function of sucrose concentration.

TABLE 4

Glucose uptake by intact cells of baker's yeast at 31°C. First group

FLASK	1		3		5		7	
ALIQUOT	S mg %	t min	S mg %	t min	S mg %	t min	S mg %	t min
0	3.32 [1]	0	16.60 [1]	0	33.2 [1]	0	88.2 [1]	0
1	3.3	12.3	15.2	11.9	30.5	11.2	84.2	10.2
2	2.9	27.8	12.7	27.3	28.1	26.7	72.2	26.0
3	2.2	48.3	10.4	48.0	24.2	47.7	60.5	47.0
4	1.7	63.7	9.0	63.2	21.4	62.4	53.6	61.7
N	1.65 n		1.65 n		1.65 n		3.30 n	

[1] Zero time concentrations were obtained by multiplying the concentration of glucose solution prepared from a gravimetric standard by the appropriate factor of dilution.

TABLE 5

Glucose uptake by intact cells of baker's yeast at 31°C. Second group

FLASK	2		4		6		8	
ALIQUOT	S mg %	t min	S mg %	t min	S mg %	t min	S mg %	t min
0	8.83 [1]	0	22.1 [1]	0	49.7 [1]	0	110.3 [1]	0
1	6.9	12.7	19.3	9.2	46.8	10.6	108.3	10.6
2	5.5	32.0	15.2	21.5	34.2	30.1	92.3	30.1
3	4.4	48.2	10.2	37.0	27.2	46.6	83.6	46.9
4	..	...	7.1	61.3	21.2	64.4	71.7	66.1
N	1.65 n		3.30 n		3.30 n		3.30 n	

$n = 1.0 \times 10^7$ cells/ml

[1] Zero time concentrations were obtained by multiplying the concentration of glucose solution prepared from a gravimetric standard by the appropriate factor of dilution.

TABLE 6

FLASK	S mols/ml $\times 10^7$	v mols/ml min $\times 10^9$	v_c mols/cell min $\times 10^{17}$
1	1.84	1.64	9.96
2	4.90	5.09	30.9
3	9.21	8.66	52.5
4	12.27	20.6	62.4
5	18.42	10.4	63.6
6	27.6	25.2	76.3
7	49.0	31.8	96.4
8	61.2	33.8	102.3

Temperature = 31°C. pH = 7.0

2. Data from the glucose uptake (hexokinase) experiments. The data obtained from experiments conducted to investigate some of the properties of yeast hexokinase by measuring the rates of glucose uptake by baker's yeast cells is presented in tables 4 and 5. Those flasks which were put on the shaker simultaneously are grouped together in the same table.

Data of tables 6 and 5 were plotted in graphical form to obtain curves of glucose concentration as a function of time for each reaction vessel. The initial slope was measured and taken as the rate, v, of glucose uptake corresponding to the initial concentration of the substrate (glucose). The rate, v, is reexpressed in mols/ml min and divided by the cell population density, N, to obtain the rate, v_c, of glucose uptake per cell. These results are given in table 6. The set of values of S and v_c, contained in table 6, is plotted in figure 2.

3. Calculation of the reaction parameters. One can use the sets of pairs of values of v_c and S given in tables 3 and 6 to compute V_{mc}, K, and β for sucrose hydrolysis and glucose uptake in the manner described earlier.

In the case of the data of table 3 for 30.5°C. the set of results as a whole is obviously not in accord with the Michaelis and Menten formulation used in the derivation of equation (5). Experimentally, a rate maximum is obtained, an effect not predicted by the simple Michaelis and Menten relationship. Probably most enzymes are inhibited by excessive concentrations of their substrate and one must therefore consider the Michaelis and Menten relation to be one that applies as a limiting law for sufficiently small values of the substrate concentration. In keeping with this notion only the ascending arm of the curve shown in figure 1 for 30.5°C. can be expected to satisfy the conditions on which the present computation is based and only that part of the data will be used for the calculation of V_{mc}, K, and β. This means that the computation, in the case of that particular set of data, will be based on only three points (see fig. 1, 30.5°C.), which, since it presents a slightly unique situation, will merit a brief discussion.

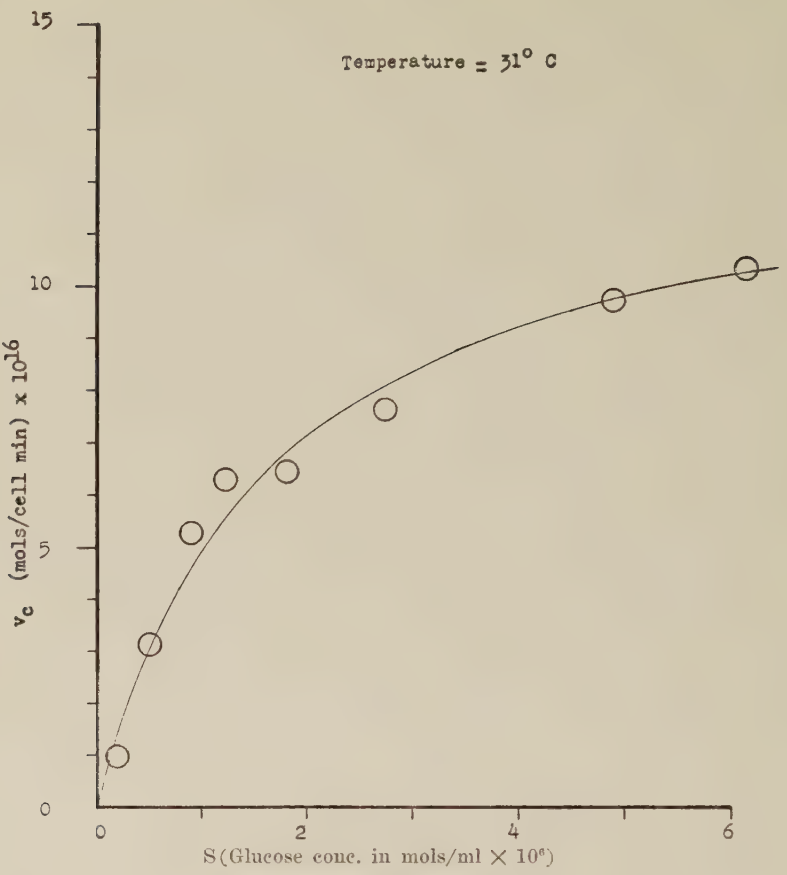

Fig. 2 Rate of glucose consumption per cell as a function of glucose concentration.

Since there are three independent parameters to be deduced from each set of pairs of v_c versus S values, at least three such pairs are required. Thus for three experimental points, in which v_c increases monotonically with S, a choice of values for the three parameters can be made so that the functional curve fits the points exactly. This means that the residual sum of squares of the fit is zero. It is therefore not necessary to carry out the minimization procedure of the non-linear regression method derived in the companion paper. One can simply take equation (5) and, by successively assigning values of the three

experimental points, obtain three simultaneous equations that are linear in the three parameters a, b, and c. These equations can then be solved for a, b, and c and hence for V_{mc}, K, and $1/\beta$. This is the procedure that was followed in the calculation of these parameters for the 30.5°C. case of sucrose.

The numerical calculation will be demonstrated in a sample calculation only for the experiment on glucose uptake (table 6). From the values in table 6 one can, in a straightforward way, obtain that

$$\Sigma v_c^2 = 373.2919 \times 10^{-32} \qquad \Sigma v_c^3 = 3086.060 \times 10^{-48}$$
$$\Sigma S^2 = 75.10269 \times 10^{-42} \qquad \Sigma v_c^4 = 2698.1495 \times 10^{-64}$$
$$\Sigma v_c S = 1568.067 \times 10^{-23} \qquad \Sigma S^2 v_c = 70.89398 \times 10^{-27}$$
$$\Sigma v_c^2 S = 1409.0401 \times 10^{-38} \qquad \Sigma v_c^3 S = 13087.571 \times 10^{-51}$$

These values can be substituted into the determinants given by relations (6)–(9). Expansion [2] by minors (see Chap. 9 of Dickson, '39) and collection of terms gives

$$|A| = -3.640 \times 10^{-118} \qquad |B| = -6.085 \times 10^{-109}$$
$$|C| = -35.29 \times 10^{-95} \qquad |D| = -32.11 \times 10^{-104}$$

so that for the hexokinase-glucose system one finds for the reaction parameters

$$V_{mc} = 1.13 \times 10^{-15} \text{ mols/cell min} = a = |A|/|D|$$
$$1.90 \times 10^{-6} \text{ mols/cc} = b = |B|/|D|$$
$$1/\beta = 1.10 \times 10^{9} \text{ cell min/cc} = c = |C|/|D|$$
$$K = 6.5 \times 10^{-7} \text{ mols/cc} = b - ac =$$

From the sucrose hydrolysis data one can compute in a similar fashion, with the exception already noted for the 30.5°C. data, that

(a) For the invertase-sucrose system at 30.5°C.

$$V_{mc} = 5.43 \times 10^{-13} \text{ mols/cell min}$$
$$1/\beta = 5.6 \times 10^{7} \text{ cell min/cc}$$

(b) For the invertase-sucrose system at 20°C.

$$V_{mc} = 2.14 \times 10^{-13} \text{ mols/cell min}$$
$$1/\beta = 1.8 \times 10^{8} \text{ cell min/cc}$$
$$K = 1.0 \times 10^{-6} \text{ mols/cc}$$

[2] This step, although a matter of straightforward arithmetic, is somewhat laborious and a desk top calculator is indispensible. A slide rule does not carry the calculation accurately to enough places to prevent cumulative computational errors from appearing in what should be the significant figures of the result.

4. Estimation of the permeability constant, h. By application of a method similar to that used for determination of "hematocrits" it was estimated that the yeast cells had a mean volume of about 2.2×10^{-11} cc., which would, with assumption of approximately spherical shape, give a mean diameter of about 3.5×10^{-4} cm and a surface area per cell of about 3.8×10^{-7} cm^2. If one uses this value for the surface area of the cell and assumes that $\beta \cong Ah$ then one obtains the following values for the permeability constant, h:

$h = 2.4 \times 10^{-3}$ cm/min for glucose at 31°C.,
$h = 4.6 \times 10^{-2}$ cm/min for sucrose at 30.5°C.,
$h = 1.5 \times 10^{-2}$ cm/min for sucrose at 20°C.

From the values of h measured for sucrose at 30.5°C. and 20°C. one can compute a $Q_{10} = 3.0$ for the permeability constant of sucrose.

DISCUSSION

1. Assumption of the steady state with regard to entry and conversion. It was assumed, under the conditions of these experiments, that the rate of entry is equal to the rate of catalytic conversion, i.e. that the steady state condition assumed in the derivation (Best, '55) of equation (4) is satisfied. Inasmuch as the external substrate concentration was steadily changing and rates were obtained by measuring the slope of the substrate-time curve at zero time, one might ask whether the steady or quasi-steady state condition is really approximated under the conditions of the experiments.

Let V be the cell volume, R the rate of change of S in the external medium at t equal zero, S_o the rate of change at t equal zero, and $k = V_{mc}/K$. It has been demonstrated in the companion paper that the steady state relationship between S and S_i will be fulfilled for practical purposes if $RV/(k + \beta) << S_o$ and $RV/\beta << S_o - v_c/\beta$ and if the time, Δt, over which the rate is estimated is very much greater than V/β.

Utilizing the data of the experiments described in this paper it was demonstrated (Best, '54) for all of the three experimental situations that each of these conditions is indeed fulfilled.

One can conclude, therefore, that the assumption of a steady state relation between S and S_i is justified.

2. Assumption of no concentration gradients. It has been assumed in the calculation of h that no appreciable gradients of the substrate were present in the interior of the cell. This assumption, along with the assumption of complete mixing of the external medium, implies that the coefficient β is just Ah, where A is the surface area of the cell. In order to examine these assumptions more closely, use is made (Best, '54) of treatments given by Rashevsky ('48, Chap. II) and Hearon ('53, I and II). Only a brief resume of the argument presented in the companion paper will be given here.

Consider the two limiting cases of a substrate whose rate of disappearance follows the Michaelis and Menten rate expression. If the substrate concentration is small, i.e. very much less than K, then its rate of disappearance, Q, will be proportional to its concentration, with V_m/K appearing as the constant of proportionality. If the substrate concentration is large, i.e. much greater than K, then $Q = V_m$ and will be independent of the substrate concentration. If one can demonstrate the absence of any contribution to $1/\beta$ by internal or external concentration gradients in each of these limiting situations, it seems clear that there will be no such contribution for any intermediate values of substrate concentration.

In the case that Q is constant for a spherical cell the assertion $\beta \cong Ah$ was shown (Best, '55) to be equivalent to the assertion

$$(1/D_e + 1/5D_i)r_o/A << 1/\beta \qquad (10)$$

when D_e and D_i are respectively the external and internal diffusion coefficients of the substrate in question and r_o and A are respectively the radius and surface area of the cell. In the case that $Q = kC$, where C is the concentration of substrate and Q its rate of consumption per unit volume at a distance r from the center of the cell, the assertion $\beta \cong Ah$ was shown in the companion paper to be equivalent to the assertion

$$(1/D_e + 1/5D_i + \xi(x)/5D_i)r_o/A << 1/\beta \qquad (11)$$

where

$$x = r_o\sqrt{V_m/KD_i} \text{ and } k = V_m/K = V_{mc}/KV$$

and $\xi(x)$ is a function for which Hearon ('53, II) has computed a table of values corresponding to various values of x.

In the case of a spherical cell it has been shown (Best, '54) that establishing the validity of the statement

$$1/\beta >> r_o/D_eA \qquad (12)$$

will mean the absence of any appreciable contribution to the diffusion resistance by possible incomplete mixing, i.e. external concentration gradients about the cell.

The value of the diffusion coefficient, D_i, for glucose and sucrose inside the cell is not known, however, from observations on the diffusion of such light molecular weight nonelectrolytes through gels one has no reason to believe (Glasstone, '46, page 1262) that D_i would differ very greatly from D_e. If D_i is much greater than D_e then the actual error would simply be smaller than that calculated by assuming them equal. In order to be on the safe side in estimating the maximum contribution likely to be incurred one can assume that D_i is only one-fifth as large as D_e. With this assumption the statements (10) and (11) and hence (12) are satisfied in the three experimental situations described in this paper. It may be concluded that, in these experiments, it is permissible to ignore the internal and external concentration gradients insofar as their contributions to the value of $1/\beta$ are concerned and β is very nearly equal to Ah.

3. Comparison with values found by other investigators. It will be worthwhile to compare the values of the permeability constant obtained here with some of those found by other investigators.

Jacobs and Stewart ('32), using an analysis of the transient volume changes induced by the penetration of various nonmetabolites, measured the values of h for a number of substances. For *Arbacia* eggs the value of h was 3.6, 5.7, 35.0, and 0.5 cm/min $\times 10^4$ for ethylene glycol, acetamid, propionamid, butyramid, and glycerol respectively.

Landahl ('39), by use of a kinetic model, computed values of h of 2×10^{-5} cm/min for lactic acid in unfertilized *Arbacia*

eggs and 25×10^{-3} cm/min for O_2 in the fertilized eggs. This latter value is about twice that of 12×10^{-3} cm/min computed by Rashevsky (p. 39, '48) from data of Gerard and Tang ('32) for O_2 penetration of fertilized *Arbacia* eggs. Landahl (Rashevsky, '48), using data of Tang ('31), finds a value of $h \simeq 12 \times 10^{-3}$ cm/min for O_2 penetration of unfertilized *Arbacia* eggs and, from data of Shoup ('29), a value of $h \simeq 2.2$ cm/min for O_2 penetration of luminous bacteria.

The values of h found in the present investigation fall well within the range of values given above by other investigators. Since none of the values given above by other investigators are for yeast one should hesitate before attaching too much significance to the agreement.

An interesting anomaly, which will be discussed a little later, is the fact that the values of h found for sucrose in the present work are larger than that of glucose.

Jacobs ('50) states that the Q_{10} for the permeability constants of different hydrophilic solutes has been found to range from 1.4 to 6.0 and that, in general, with the same cell, the temperature coefficient tends to increase with increasing molecular weight of the penetrating substance. In the light of this one can consider the Q_{10} of 3.0, found for the h of sucrose, to be well within the range of values customarily encountered.

4. Applications to cytological inference. In the cell membrane model of Danielli (Davson and Danielli, '43, Appendix A) or Zwolinski et al. ('49), the molecules of a substance diffusing across the cell membrane are confronted by one or more free energy barriers. If the substance is water soluble, fat insoluble, and encounters a greater diffusion resistance in passing through the membrane than through a water layer of comparable thickness, then a larger activational energy is involved in the membrane transition than in the process of ordinary diffusion through water. Suppose an enzyme, as a cellular component, does not actually lie within the envelope of the cell membrane but lies instead upon the surface of the cell, outside the region enclosed by the membrane proper. If the mixing of the medium containing the cell and substrate were

perfect, i.e. extremely rapid in comparison to the rate of reaction, one should measure a zero value for 1/Ah of the substrate being consumed by the enzyme *in situ*. Since perfect mixing is not, in practice, attainable, one would in general find a small but non-zero value for 1/Ah in such a situation. With a sufficient number of values for 1/Ah for a wide enough variety of other substances entering the cell, then, by comparison, one might tell whether a particular value of 1/Ah could be considered anomalously low. At the present stage such measurements are not sufficient or extensive enough to render this approach feasible. However, two methods of circumventing this difficulty will be presented in the discussion to follow.

If in the process of transition, from the external medium to the site of location of the enzyme in the cell, the substrate molecule must pass over a sizeable free energy barrier such as would be presented by a membrane, then one would anticipate an appreciable temperature dependence of h and hence of 1/Ah. If, on the other hand, the substrate did not have to penetrate any actual membrane to get to the enzyme, then one might anticipate a rather low temperature dependence of 1/Ah approximating that of the temperature dependence of the diffusion coefficient of the substrate through water.

This discussion has embraced two simplifying assumptions the validity of which one might question. The surface area was not assumed to be appreciably temperature dependent and the enzyme was assumed to be either interior to the cell membrane or on the outermost perimeter of the cell.

Suppose that one carried out a measurement of the formal value of $1/\beta$ for a substrate B that is being consumed by an enzyme which one has reason to believe, by independent experimental evidence, lies interior to the cell membrane. Let us also suppose that one measures a value of $1/\beta$ for a substrate C of similar but not identical chemical structure for which the location of the corresponding enzyme is in doubt. If the unknown enzyme lies outside the cell membrane, then one would expect to find an appreciably smaller formal value of

$1/\beta$ for C than for substrate B. If, on the other hand, the enzyme, whose position is in question, lay within the envelope of the membrane proper, then one might reasonably expect that the value of $1/\beta$ for substrate C would approximate that for B. If the structure of C is such that it would have greater difficulty of penetration than B and is found to have an appreciably smaller value of $1/\beta$ than B, then it might reasonably be concluded that C's enzyme lay outside the membrane enclosing B's enzyme.

The formal properties of $1/Ah$ (and also $1/\beta$) are very similar to those of resistance in electrical theory. If one wishes to consider concentration differences as analogous to potential difference and the area multiplied by the flux of material as analogous to the current, then $1/Ah$ and $1/\beta$ will be analogous to resistance. $1/Ah$ and $1/\beta$ have the desirable property that their values for a multi-layered barrier is just the sum of such quantities for the individual layers. This is not characteristic of β or h. The quantity $1/\beta$ will be termed the "desistance" and, in the light of the arguments of part 2 of this section, the term will also be applied to the formal value of $1/Ah$.

With the preceding arguments in mind one can proceed to draw some inferences from the data obtained in the experiments described in this paper.

5. Some conclusions regarding the cytological arrangement of invertase and hexokinase in yeast and a discussion of those conclusions in the light of some of the findings of other investigators. Although it will be shown later to be inadequate to explain all of the facts let us adopt, for purposes of argument, the simple conception that an enzyme, as a cellular component, must either be interior or exterior to the cell membrane. Consider the particular situation presented by invertase and hexokinase of baker's yeast. The diffusion coefficient of glucose in water is about 25% greater than that of sucrose, therefore, if both invertase and hexokinase lay exterior to the cell membrane, on the cell surface, one would expect the desistance measured for glucose to be approximately 25% less

than that for sucrose. This was not the case. The desistance for glucose was found to be approximately 18 times that of sucrose at the same temperature. One can also discard the possibility that both invertase and hexokinase are inside the membrane. Since sucrose has a larger number of OH groups and molecular size than glucose, one would expect sucrose to have greater difficulty in penetrating the cell membrane than glucose. If both enzymes were inside the membrane there would be an appreciably larger desistance for sucrose than for glucose. Since the converse is true one must discard the possibility that both enzymes are interior to the cell membrane.

Two remaining permutations of this simple model are possible. Hexokinase could be outside and invertase inside or hexokinase inside and invertase outside. Continuing the line of argument of the preceding paragraph, one is forced to conclude that hexokinase must be interior and invertase exterior to the cell membrane.

Having reached the conclusion that some kind of barrier to diffusion must be interposed between the cellular locations of invertase and hexokinase one must now ask whether there is more to the matter. If there is only a single diffuson barrier of negligible thickness called the membrane and invertase lies outside of this then it must be concluded that the only contribution to the formal value of the desistance of sucrose arises from stagnant water layers in the immediate neighborhood of the cell through which sucrose must diffuse to get to the cell surface. Two pieces of evidence can be aligned against this view. First is the fact that it has already been shown in part 2 of this section that the desistance for sucrose is sufficiently large that even if no mixing were occurring the contribution by external concentration gradients would be negligible. The second objection is that the Q_{10} of 3.0, which was found for the desistance of sucrose, is larger than that which would be anticipated from the temperature dependence of the aqueous diffusion coefficient of sucrose. There would appear to be some kind of structural barrier to diffusion interposed between invertase and the external medium and since in-

vertase must lie outside the membrane proper one must conclude that the structural components representing this secondary barrier to diffusion must extend even farther beyond the confines of the membrane proper.

One can, therefore, propose the tentative model illustrated schematically by figure 3 and ask the extent to which it agrees with the findings of other investigators. Because of space limitations only a few of the corroborative findings of other

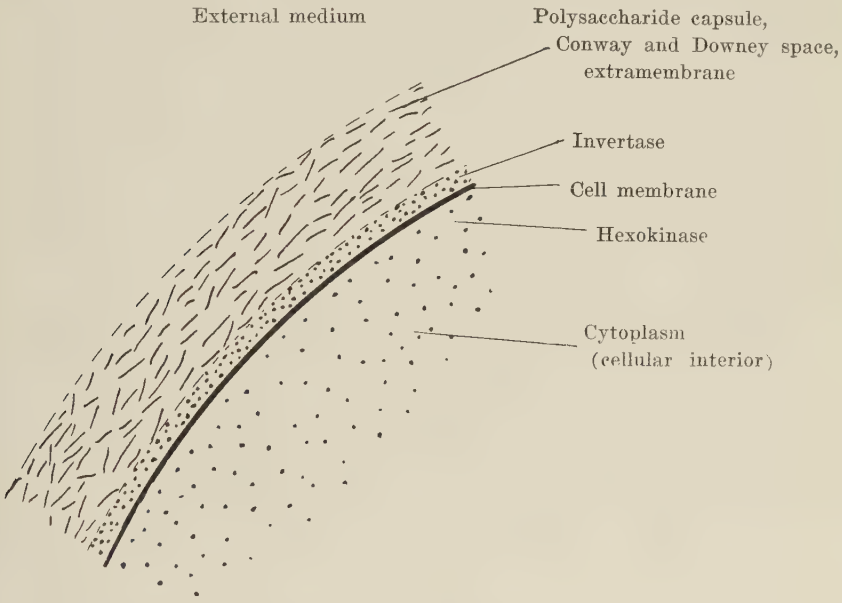

Fig. 3 A tentative model for the cell surface of baker's yeast.

investigators can be indicated (for a more complete discussion see Best, '53).

The conclusion that the cell surface is the primary site of diffusion resistance of the substrate into the cell is in agreement with the findings of Cole and Curtis ('38, '39) and Cole ('37) that it is the primary site of electrical resistance to low frequency or direct currents.

In plant cells, aside from internal deposits of starch or glycogen, probably the bulk of the polysaccharides, such as

dextrans, levans, celulose, etc., are outside the membrane proper either conjugated to the surface proteins of the cell or in the form of a thick capsule. Invertase separates out with the polysaccharide fraction of yeast extracts and seems to be a microprotein tightly conjugated to a relatively large polysaccharide component (Neuberg and Mandl, '50). It would not be surprising, therefore, to find invertase in the external polysaccharide capsule. A porous external structure in yeast seems to be indicated by the investigations of Conway and Downey ('50) from a comparison of hematocrit volume with the inulin space of dense yeast suspensions. They conclude that approximately 10% of the yeast cell volume must be outside the cell membrane.

Barron at al. ('48), from a well planned study of the inhibitory effects of the uranyl ion on yeast, conclude that hexokinase is interior to the cell membrane and invertase probably exterior to it. In agreement are the findings of Wilkes and Palmer ('32), who, from studies of pH dependence of the rate of catalysis, furnish circumstantial evidence that invertase is at the cell surface, and Derrick, Miller and Sevag ('53), who, using immunological techniques, have produced persuasive evidence that hexokinase of baker's yeast is interior to the cell membrane. Myrback and Vasseur ('43), from a pH dependence study similar to that conducted by Wilkes and Palmer, conclude that the saccharases, lactase and trehalase are at the cell surface.

The results of the present study are interesting in that they support the findings of the previous investigations cited by means of an independent set of experiments and considerations.

SUMMARY

When a catalyst is enclosed by a barrier, such as a cell membrane, to the diffusion of the substrate that is undergoing catalytic transformation, the measurement of the reaction parameters characterizing the catalyst must be carried out simultaneously with the measurement of the permeability of

the barrier to the substrate. A minimal model was described for examination of kinetic data obtained on intact cells. This model considers a catalyst, showing Michaelis and Menten dependence of rate on substrate concentration, that is constrained within a membrane-like barrier to diffusion of the substrate.

In a companion paper (Best, '55), several methods were derived for evaluating the barrier parameter, β, and the catalytic parameters, K and V_{mc}, from a set of pairs of values of external substrate concentrations, S, and the rate of consumption per cell, v_c. One of these, the non-linear regression method, was given in summarized form in this paper.

The rate of hydrolysis of sucrose by intact cells of Fleischman's baker's yeast at a series of substrate concentrations was investigated at 30.5 and 20°C. The rate of glucose consumption by intact cells of this yeast was measured for each of a series of glucose concentrations at 31°C. Values of β, K, and V_{mc} were estimated for each of these three sets of data by means of the non-linear regression method. By means of measurements of the cell volume and the assumption of approximately spherical shape for the yeast cells, the surface area, A, was estimated. This estimate of A, along with the estimates of β, were used to obtain values for the membrane permeability, h. A Q_{10} of 3.0 was computed for the h of sucrose. The estimates of h and its temperature dependence were found to fall well within the range of values reported by other investigators for other substances and cells.

In the formulation of the kinetic model it had been assumed that the relationship between the external and internal substrate concentrations was that given by a steady state with regard to entry and catalytic transformation of the substrate. A brief resume of the manner of investigation of this assumption, carried out in more detail in the companion paper, was given. It was concluded that the steady state assumption was justified in the case of the experiments described in this paper.

It is possible, by using some of the consequences of treatments by Rashevsky ('48) and Hearon ('53), of diffusion and

catalysis in a spherical cell, to answer two questions regarding the present work. First, can insufficient rates of mechanical mixing give an appreciable contribution to the value of the desistance, $1/\beta$, by permitting concentration gradients of the substrate to be formed in the external medium in the neighborhood of the cell? Second, is there an appreciable contribution to the value of $1/\beta$ due to the presence of internal concentration gradients of the substrate? A brief resume of the discussion of these questions in the companion paper was given and the answer to both questions found to be negative with regard to the experiments described in the present paper.

Arguments concerning the cytochemical interpretation of the relative values obtained for the desistance, $1/\beta$, were presented which seemed to suggest that hexokinase was inside the membrane proper of the yeast cell and invertase outside. Further arguments were presented that indicated the existence of some kind of lesser barrier interposed between invertase and the external medium. Some of the work of other investigators that would lend support to these conclusions was cited.

The author wishes to thank Dr. John Z. Hearon of the Mathematics Panel of the Oak Ridge National Laboratory for his constructive criticisms of the theoretical portions of this work and Doctors Daniel Harris and John O. Hutchens of the Department of Physiology of the University of Chicago for their valuable suggestions regarding certain aspects of the experimental procedure.

LITERATURE CITED

BARRON, E. S. G., J. A. MUNTZ AND B. GASVODA 1948 Regulatory mechanisms of cellular respiration. I. The role of cell membranes: Uranium inhibition of cellular respiration. J. Gen. Physiol., *32:* 163.

BEST, JAY BOYD 1953 Doctoral dissertation. Department of Physiology, the University of Chicago.

——— 1955 The inference of intracellular enzymatic properties from kinetic data obtained on living cells. I. J. Cell. and Comp. Physiol., *46:* 1.

COLE, K. S. 1937 Electric impedance of marine egg membranes. Transac. of the Faraday Soc., *33:* 966.

COLE, K. S., AND H. J. CURTIS 1938 Electrical impedance of Nitella during activity. J. Gen. Physiol., *22:* 37.

———— 1939 Electrical impedance of the squid giant axon during activity. J. Gen. Physiol., *22:* 649.

COLLANDER, R., AND H. BARLUND 1933 Permeabilitätsstudien an Chara Ceratophylla. II. Die Permeabilität für Nichtelektrolyte. Acta Bot. Fenn., *11.*

CONWAY, E. J., AND MARY DOWNEY 1950 An outer metabolic region of the yeast cell. Biochem. J., *47:* 347.

DAVSON, H., AND J. F. DANIELLI 1943 The Permeability of Natural Membranes. Appendix A. Univ. Press, Cambridge.

DERRICK, M. J., R. E. MILLER AND M. G. SEVAG 1953 Yeast phosphatase. Immunological method of determining its location in the cell. Fed. Proc., *12:* 196.

DICKSON, L. E. 1939 New First Course in the Theory of Equations, Chap. 9. John Wiley and Sons, Inc., New York.

GLASSTONE, SAMUEL 1946 Textbook of Physical Chemistry. 2nd ed. Page 1262. D. van Nostrand Co., Inc.

HEARON, JOHN Z. 1953 Comments on the approximate solution of the diffusion equation. I. Bull. Math. Biophysics, *15:* 23.

———— 1953 Comments on the approximate solution of the diffusion equation. II. Bull. Math. Biophysics, *15:* 111.

JACOBS, M. H. 1930 Osmotic properties of the erythrocyte. I. Biol. Bull., *58:* 104.

———— 1950 Surface properties of the erythrocyte. Ann. N. Y. Acad. Sci., *50:* 815.

JACOBS, M. H., AND D. R. STEWART 1932 A simple method for the quantitative measurement of cell permeability. J. Cell. and Comp. Physiol., *1:* 71.

LANDAHL, H. D. 1939 Mathematical biophysics of cell respiration. II. Bull. Math. Biophysics., *1:* 1.

MICHAELIS, L., AND M. L. MENTEN 1913 Die Kinetik der Invertinwirkung. Biochem. Z., *49:* 333.

MILLER, B. F., AND D. D. VAN SLYKE 1936 A direct microtitration method for blood sugar. J. Biol. Chem., *114:* 583.

MYRBACK, K., AND R. VASSEUR 1943 Über die Lactosegärung und die Lokalisation der Enzyme in der Hefezelle. Zeit. Physiol. Chemie, *277:* 171.

NEUBERG, C., AND I. MANDL 1950 Invertase. The Enzymes, edit. by Sumner and Myrback, Vol. I, part I, Chap. 14, 527.

PARPART, A. K. 1935 The permeability of the mammalian erythrocyte to deuterium oxide. J. Cell. and Comp. Physiol., *7:* 153.

RASHEVSKY, N. 1948 Mathematical Biophysics. rev. ed. University of Chicago Press, Chicago.

RUHLAND, W., AND G. HOFFMAN 1925 Die Permeabilität von Beggiatoa Mirabilis. Ein Beitrag zur Ultrafiltertheorie des Plasmas. Planta, *1:* 1.

SHOUP, C. 1929 The respiration of luminous bacteria and the effect of oxygen tension upon oxygen consumption. J. Gen. Physiol., *13:* 27.

Somogyi, M. 1945 Determination of blood sugar. J. Biol. Chem., *160:* 69.

Tang, P. S. 1931 The oxygen tension consumption curve of unfertilized Arbacia eggs. Biol. Bull., *60:* 242.

Tang, P. S., and R. W. Gerard 1932 The oxygen tension-oxygen consumption curve of fertilized Arbacia eggs. J. Cell. and Comp. Physiol., *1:* 503.

Wilkes, B. G., and Elizabeth T. Palmer 1932 Similarity of the kinetics of invertase action in vivo and in vitro. II. J. Gen. Physiol., *16:* 233.

Zwolinski, Bruno J., H. Eyring and C. E. Reese 1949 Diffusion and membrane permeability. I. J. Physical and Colloid Chem., *53:* 1426.

ON THE OPTICAL CHANGE ASSOCIATED WITH ACTIVITY IN FROG NERVE

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During the last 5 years or so it has been demonstrated that the light scattered by a multifibered nerve in a beam of white light changes as a function of impulse propagation (Hill and Keynes, '49; Hill, '50, '50a; Bryant and Tobias, '52). Early experiments were all done using marine invertebrate nerves, and since the effect was not at first detected with frog nerve it appeared that it might occur only in certain forms and therefore be of limited interest. Also, if the phenomenon were thus restricted then work on it might be limited to marine station excursions or to experiments with animals shipped over long distances. It was of interest therefore that the phenomenon was soon found also to occur with frog nerve, but it was not further investigated in the frog at the time (Bryant and Tobias, '52). The present communication is devoted primarily to a more detailed description of the frog nerve optical response, to the influence upon it of certain variables and to some experiments which have bearing on interpretation.

It should be made clear that the experiments have been done in awareness of the fact that it is not yet known how individual axons contribute to the whole nerve optical response. No change in scattering has yet been reported to accompany activity in single isolated axons. Therefore the change in

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light scattering seen with bundles of axons or with whole nerves may be due to spatial redistribution of the axons, to changes in their form and size, to changes in interfibrillar material or to all of these, and may not reflect any impulse-critical change in axonal structure *per se*. The present experiments thus relate directly, so far as we are now certain, to bundles of axons or to whole nerves. One may learn later what they mean in terms of more subtle axonal structure, but this is not yet clear.

METHODS

The photoelectric equipment used was essentially that described by Bryant and Tobias ('52). It has, however, been more convenient to record with an ink-writing milliammeter than manually as before from galvanometer readings. In addition, to eliminate twisting of the nerve, the silk thread tied to each end, instead of being pulled through a small, cork filled hole in the chamber cover, was passed through a slit and held by a miniature battery clip (fig. 1 a). Other modifications of the chamber for particular experiments will be noted in the text. White light was used throughout. In all cases the light measured was that scattered through an angle of 90° to the incident beam.

Stimulation and detection equipment was conventional.

Normal Ringer solution, brought to pH 7.4 with phosphate buffer, contained NaCl 6.5 gm, KCl 0.14 gm, $CaCl_2$ 0.12 gm and water to 1 litter.

All experiments were done with the frog sciatic nerve.

Statistical evaluations were made in terms of standard error where

$$\text{S. E.} = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

Reservations concerning the use of the term "scattering" in these experiments have been discussed before (Bryant and Tobias, '52). They still apply.

RESULTS

Description of the commonest optical response

When frog nerve, whether desheathed or with sheath intact, is put into Ringer solution one observes a resting drift in light intensity. At first, when the incident beam is larger in cross section than the nerve itself, the drift is toward in-

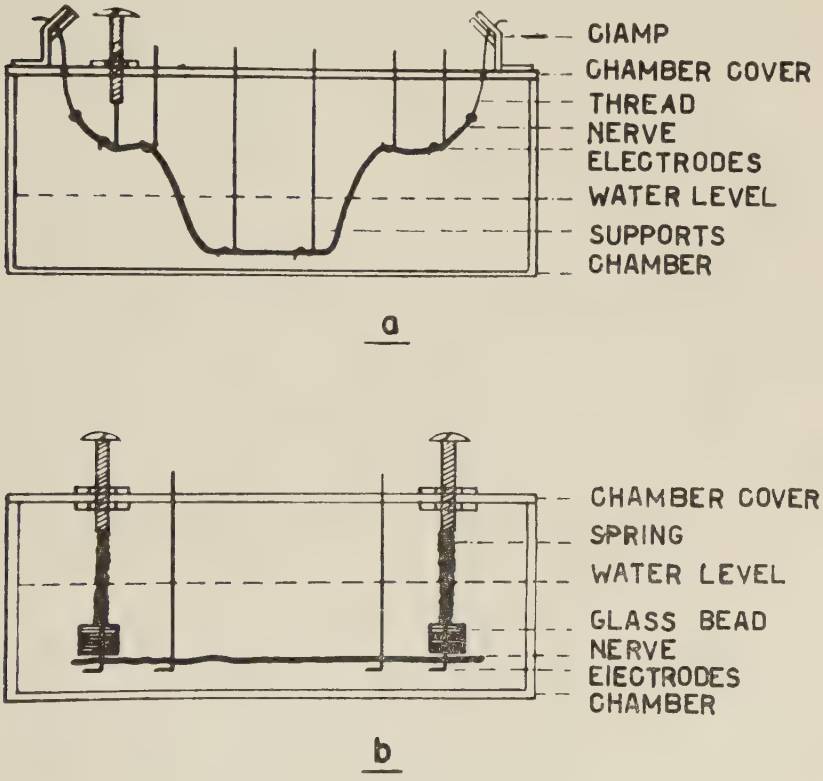

Fig. 1 Alternative arrangements of nerve in chamber.

creasing intensity, but this decelerates, and after a variable time, *ca.* 30–80 minutes, the scattered light in most cases decreases in intensity.

An additional change occurs with stimulation, being of the form shown in figure 2. First there is an apparent latency

which is followed by a decrease in scattered light. This decrease has been fairly linear for stimulation periods up to 90 seconds. Cessation of electrical activity is followed by optical overshoot and finally there is return toward normal. Recovery is variable and often incomplete. The response is qualitatively much like that seen with unstretched Carcinus nerves (Bryant and Tobias, '52).

Table 1 gives numerical information about the several parts of the response, using desheathed nerves mounted as in figure 1 a. Apparent latency averaged about 6.3 seconds.

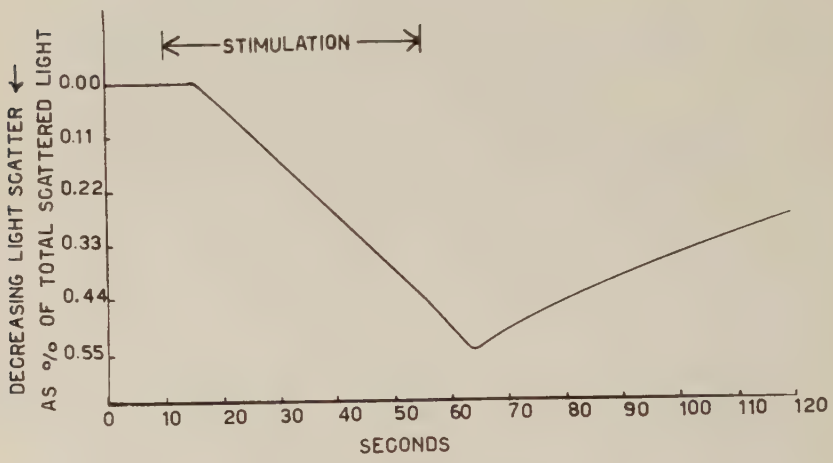

Fig. 2 Representative optical response of frog nerve.

It is not yet clear whether this is a true latency or whether it is an interval occupied by opposite and balancing optical changes. By interpolation, the average rate of scattering decrease, following the delay, was $0.00098 \pm 0.00016\%$ of the total initially scattered light, per second. The optical overshoot averaged 7.9 seconds and amounted to about 18.3% of the change during stimulation.

Recovery times were difficult to assess. Sometimes, in 4 cases out of 47 in table 1, essentially no recovery could be detected. In others the scattered light intensity returned only incompletely to the resting level. However, if recovery

TABLE 1

Optical responses by desheathed frog nerves

(Nerves mounted as in figure 1a, bent and partially immersed. All data averages)

NO. OF STIM'N. PERIODS	LATENCY OF ONSET, SECONDS	DECREASE IN SCATTERED LIGHT			RECOVERY			OVERSHOOT	
		Total % change	% change per second	S.E.	Number showing no recovery	Recovery no.	Recovery sec.	Duration, seconds	As per cent of change during stim'n.
5	6.0	0.042	0.00094	0.00004	2	3	288	6.6	9.7
8	5.3	0.029	0.00065	0.00011	0	3	340		
						5	60	7.5	19.3
9	7.6	0.042	0.00095	0.00003	1	8	136	8.3	27.7
8	7.9	0.033	0.00074	0.00003	1	7	132	10.1	20.0
7	4.3	0.042	0.00097	0.00006	0	7	133	7.0	20.0
10	6.6	0.073	0.00162	0.00002	0	10	406	7.8	11.5

Temperature fluctuated with room temperature.
Recovery time extrapolated from recovery rate during first 30 seconds after end of overshoot.
Stimulation in all cases supramaximal at 430 shocks per second for 45 seconds.

were to continue at the same rate as during the first 30 seconds following the end of overshoot then it would be complete in about 215 seconds. Thus recovery is considerably slower than development of the change, and it is probably underestimated by the figure computed.

Reproducibility of responses by a given nerve is illustrated by tables 1 and 2. Thus, table 1 shows (temp. variable, 22.8–31.2°C.) that, after correction for basal drift, the responses by any one nerve were reproducible with a standard error of about 6% of the mean: table 2 shows that with constant temperature the average standard error was about 10.2% of the mean.

TABLE 2

Reproducibility of responses at constant temperature

(Nerves mounted as in figure 1b, straight and fully immersed)

NUMBER OF STIMULATION PERIODS	PER CENT DECREASE IN SCATTERED LIGHT PER SECOND	S.E.
4	0.0035	0.0003
4	0.0029	0.0003
5	0.0035	0.0004
4	0.0048	0.0004
4	0.0024	0.0003

All nerves stimulated supramaximally at 430 shocks per second for 45 seconds

The light source

The light source itself could influence scattering and scattering changes by the illuminated nerve, either thermally or photochemically.

While it seems reasonable that the optical response will exhibit a certain thermal dependency, no such relationship has become apparent between 22.8 and 31.2°C. Nor were the responses from a given nerve more reproducible when temperature was held constant. Therefore the phenomenon must have a rather small temperature coefficent. Thermal changes within the nerve itself have not been adequately measured, but in one experiment a nerve was wrapped around a ther-

mocouple and put in the chamber in the light beam. The temperature rose from 23.0 to 28.4°C. in 153 minutes, a rise similar to that in the bulk of the medium in many experiments.

Experiments were also done in which light reached the nerve intermittently. The resting drift was roughly the same, and the drift reversal was seen as usual whether the light was on periodically for a total of 5% of the time or continuously for 100% of the time. It also made little difference

TABLE 3

Optical response with light on continuously or intermittently

NERVE	DECREASE IN SCATTERED LIGHT INTENSITY WITH LIGHT ON ONLY BEFORE AND AFTER STIMULATION (ARBITRARY UNITS)		DECREASE IN SCATTERED LIGHT INTENSITY WITH LIGHT ON CONTINUOUSLY (ARBITRARY UNITS)	
	Time after excision	Response	Time after excision	Response
	min.		*min.*	
	39	73		
1	46	80		
	51	84		
			56	64
	158	55		
2	169	52		
	175	66		
			182	64

Stimulation supramaximal at 430 shocks per second for 45 seconds

to the activity response whether the light was on continuously or only long enough for measurements to be made just before and just after stimulation (table 3).

Therefore, white light, as used, had little if any effect on the resting optical drift or on the optical response.

Bending of the nerve, extent of immersion, tension and dilution of medium

Since nerves have usually been mounted with their ends out of the fluid medium, to facilitate stimulation and impulse

TABLE 4

Effect of nerve mounting

Nerve bent and partially immersed Figure 1a		Nerve straight and totally immersed Figure 1b	
Time after excision	Decrease in scattered light (arbitrary units)	Time after excision	Decrease in scattered light (arbitrary units)
min.		*min.*	
35	28, 30	45	36, 36, 28, 22, 20, 20, 22, 22
45	20, 12, 26, 20, 16	45	34, 38
45	34, 28	60	26, 32, 24, 22, 34
45	26, 28, 18, 28	60	26, 34, 40, 30, 28, 34
30	28, 26, 34, 26, 18	70	26, 28, 36
50	36, 34, 34	75	40, 42, 38, 30, 38, 34, 44, 32, 42
90	12, 30, 14, 24	180	40, 36, 44, 38, 34
45	26, 28, 22		
1040	22, 16, 32, 14		
	mean 25 S.E. 1.19		mean 32.4 S.E. 1.10

Each set of data is from a separate nerve. In all cases stimulation was supramaximal at 430 shocks per second for 45 seconds.

detection, there has been a certain amount of distortion and lateral compression against the electrodes and supports (fig. 1 a). Because the resting drift and optical response of crab nerve are both sensitive to tension (Bryant and Tobias, '52), is seemed desirable to examine the mounting technique for effects on the frog nerve optical response also. Nerves were therefore mounted bent and partially immersed or straight and fully immersed (fig. 1). Table 4 shows that the optical responses were in the same direction in both cases. Quantitatively however the responses were somewhat greater with the fully immersed, straight nerve. For quantitative work therefore it is probably important to consider the mode of mounting.

Anticipating therefore that tension would reverse the response in frog nerve as it did with Carcinus nerve, longitudinal tension was progressively increased, in 4 experiments, after observing initial responses with the nerves lax. In no case was the response reversed. The only consequence of such treatment was a diminution of the response as the nerve was stretched, probably due to block of an increasing number of fibers.

With crab nerves it was found that hypotonicity could also reverse optical response direction (Hill, '50; Bryant and Tobias, '52). With the frog nerve however, dilution of the medium, by adding from less than 30 up to 80% distilled water to the Ringer solution had no effect. The only observable change again was a decrease of the response. The resting shift, on the other hand, was markedly affected by dilution. In all cases (4 desheathed and 12 intact nerves) hypotonicity caused the resting drift to change from increasing to decreasing light scatter.

Since these experiments were finished, we have, on occasion, seen frog nerve show increased light scattering with activity. This has, however, been a random occurrence, and we are not able to ascribe it to any particular experimental condition.

Desheathing

The connective tissue sheath of the frog sciatic nerve might be expected to attenuate the optical response. If the sheath contributes to the total light scattered but does not itself change with activity then the percent change brought about by the active elements should be smaller than if the sheath were removed. Therefore responses of desheathed and intact nerves were compared.

When this was done no significant difference was found between total light scattered by the two preparations (48 nerves). Nevertheless the optical responses were, on the average, larger with desheathed nerves (table 5). Since the desheathed nerves had usually been excised for a longer time than the intact ones before they were used, the optical response difference might somehow have reflected this aging difference. However, there was considerable overlap so this probably was not a factor.

One then wonders why desheathing increased the response even though it did not decrease the total scattering light. A tentative explanation follows: Upon desheathing it can be seen that bundles of axons herniate through the incision and spread out some as they are freed from restraint. Because of the initial dense packing such spread probably increases the light scattered per bundle, since more parts of more bundles are exposed to the incident beam. Therefore desheating brings axons and axon bundles into the effective optical system which were previously shadowed. In addition, a larger area of the incident beam is intercepted by the partially "exploded" nerve. Thus the total scattered light stays about the same when the sheath is removed, but now a greater portion of the light is scattered by active elements than before when the sheath was present and also scattering. Therefore the activity response might be expected to increase, as it did.

TABLE 5

Effect of desheathing

(Mounted as in figure 1a)

SHEATH INTACT			SHEATH REMOVED		
Time after excision	Responses recorded, no.	Av. response, arb. units	Time after excision	Responses recorded, no.	Av. response, arb. units
min.			*min.*		
37– 44	2	29	39– 44	2	43
38– 67	5	26.4	54– 62	2	33
46– 83	5	18.2	184–200	5	39
48– 78	4	25	187–215	8	31.5
49– 64	4	27.5	188–212	7	29.7
53– 60	2	31	188–224	10	73.3
53– 73	3	25.3	191–215	7	38.3
60– 77	3	34.7	192–224	9	33
95– 115	4	17	621–648	5	40.5
1023–1052	4	21			
	overall average	25.5		overall average	40.1

Light scattering, nerve water content and the effect of limiting the incident beam

It has been mentioned that frog nerve put into Ringer solution increases its light scattering for some time, the lax Carcinus nerve behaving similarly in sea water (Bryant and Tobias, '52). Since frog nerve is known to gain weight in Ringer solution, and since cellular-extracellular water shifts have been emphasized as playing an important role in light scattering changes with activity (Hill, '50, '50a), experiments

TABLE 6

Resting drift of scattered light and nerve weight gain

(Nerves in normal Ringer. Incident beam larger than nerve cross section)

DURATION OF DRIFT MEASUREMENT	DRIFT, I.E. INCREASE IN SCATTERED LIGHT AS PER CENT OF TOTAL INITIALLY SCATTERED LIGHT	WEIGHT GAIN DURING OBSERVATION		
		Initial wt.	Final wt.	Gain
min.		*mg*	*mg*	*%*
19	1.5	16.8	19.3	15
25	4.9	19.0	21.0	10.5
29	9.5	17.4	18.1	4.1
55	2.0	23.6	24.1	2.1
119	10.6	22.8	26.9	18.1
130	9.6	21.6	24.7	16.7
150	14.2	17.8	23.3	31.2
145 [1]	9.3 [1]	15.3 [1]	16.5 [1]	8.2 [1]

[1] All nerves desheathed except the last one.

were done to determine if the above two events, weight gain and increasing light scatter, are associated in time. Table 6 shows that weight gain by nerves in Ringer solution is associated with an increase in scattered light.

The findng is interpretable if the weight gain is due to extrafibrillar fluid uptake as is the case with desheathed bullfrog nerve (Shanes, '53), the argument being the same as in the preceding section. Thus scattering increase is due to increased spatial separation and spread of axons and axon bundles attendant upon interfibrillar water intrusion. This would unshadow and expose more axon bundles and axons

and wound enlarge the whole nerve cross section. Both factors should bring more of the incident light into the system and, as was observed, should therefore increase the total amount of scattered light. Now, if the above is correct then limiting the incident beam so as to make it smaller in area than the nerve should modify the light scattering and light scattering changes in an understandable manner.

Experiments were therefore done using a limited incident beam (fig. 3). The nerve was mounted on a curved plastic shelf behind a glass plate fixed to the chamber cover. The shelf was cut out in two places so as to allow downward

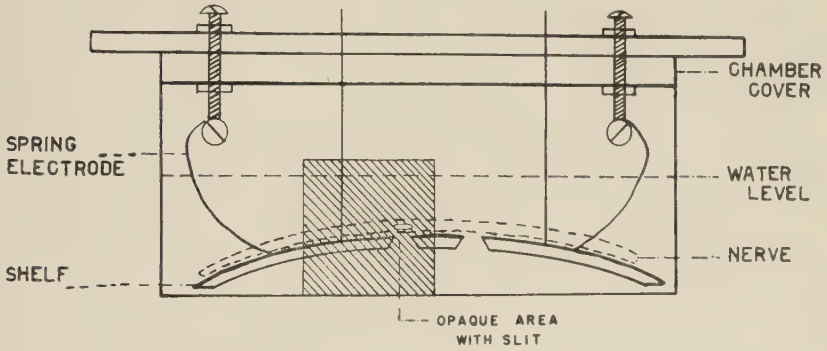

Fig. 3 Chamber for limiting incident beam.

scattered light to reach the detector photocell with no more absorption than in previous experiments. The glass in front of one of these regions was left transparent whereas in front of the other it was painted black except for a fine slit smaller than the nerve. The paint was on the rear surface of the glass so that the nerve could be brought into contact with the slit thus avoiding spreading of the incident beam before reaching the nerve. Table 7 shows measurements of the resting drift with this arrangement.

Five nerves examined with the restricted beam showed decreasing light scattering even though they all gained weight. This is the opposite of what had previously been observed with the unrestricted beam (table 6). Of 9 nerves examined

TABLE 7

Effect of incident beam width on resting drift

(In Ringer solution)

INCIDENT BEAM UNRESTRICTED			INCIDENT BEAM LIMITED TO NERVE			WEIGHT GAIN
Duration of observation	Change in scattered light	Direction of change in scattered light	Duration of observation	Change in scattered light	Direction of change in scattered light	
min.	%		*min.*	%		%
			60	4.1	decrease	1.2
			24	5.5	decrease	7.1
			135	13.2	decrease	10.6
			60	7.3	decrease	5.9
			48	5.0	decrease	4.1
15	1.5	increase	15	2.2	decrease	
15	0.3	increase	15	2.6	decrease	5.2
14	2.6	increase	10	7.0	decrease	
13	0.8	increase	13	4.4	decrease	5.2
14	1.8	increase	13	0.2	decrease	
14	0.3	increase	15	0.2	decrease	7.8
13	0.7	increase	15	2.9	increase	
13	1.3	increase	14	0.9	increase	1.9
11	3.8	increase	15	2.9	increase	
15	0.1	increase	15	0.4	increase	7.2
14	1.0	increase	13	0.6	decrease	5.3
15	1.3	increase	11	2.9	increase	8.5
13	1.1	increase	12	10.8	decrease	5.2
14	1.4	increase	15	1.7	increase	10.5

All nerves desheathed except number 1.

alternately with unrestricted and restricted beams, 5 showed drift reversal and 4 did not. The data are therefore erratic, but it is felt that they support the hypothesis outlined. Failure to show reversal in some cases was probably due to inaccurate positioning of the nerve on the slit. This was done by eye, and was not under perfect control, particularly since after positioning the nerve the whole assembly had to be lowered into the fluid filled chamber. If the nerve moved

TABLE 8

Effect of incident beam width on scattering change with activity

INCIDENT BEAM UNRESTRICTED		INCIDENT BEAM LIMITED TO NERVE	
Number of stimulation periods	Decrease per second in total scattered light	Number of stimulation periods	Decrease per second in total scattered light
	%		%
3	0.0017	2	0.0041
2	0.0022	2	0.0055
2	0.0019	2	0.0039
4	0.0023	5	0.0042
4	0.0040	4	0.0070
4	0.0032	3	0.0029
6	0.0028	3	0.0029
6	0.0027	5	0.0039
6	0.0030	3	0.0043
4	0.0044	4	0.0059
	mean 0.0028		mean 0.0044

All stimulation supramaximal at 430 shocks per second for 45 seconds.

only very little and did not entirely cover the slit the system could act qualitatively as if there were no slit present at all. It would appear, therefore, that with the restricted beam some of the nerve elements go out of the beam when it swells, and, this being enough to compensate for unshadowing due to water entry, the total amount of scattered light decreases.

Because of these findings it was anticipated that limiting the incident beam would increase the activity response. Table 8 shows that in 9 cases out of 10 this expectation was realized.

Two points deserve special attention: (A) If such work is ever to yield vigorously defensible quantitative data concern-

ing a change in molecular light scattering in nerves, axons or any other cells then the total amount of light entering the system, i.e. the effective illumination area, as well as intensity must be known and controlled, a difficult matter at high sensitivities when nerve dimensions and packing are changing during the measurements. As it is now, with a large incident beam the nerve may swell to intercept more light: with a small beam the nerve may swell beyond it: similar changes of opposite sign would occur if the nerve were shrinking. Whether the beam be large or small, separation of nerve elements, as by water intrusion can, if the system is dense to begin with, expose more elements to the incident light, and thus dilution can produce an apparent increase in scattered light. On the other hand, in a region such as the axoplasm where ultramicroscopy has shown light scattering to be minimal as compared to surface regions (Tobias and Bryant, '55) dilution might very well have no effect at all on light scattering if the beam is relatively large. The experiments clearly point up procedural parameters of importance.

(B) It has been suggested that the scattering decrease seen with activity is somehow associated with increased axonal hydration (Hill, '50, '50a). The fact that Ringer solution uptake by frog nerve is associated with a scattering increase rather than with a decrease is not in conflict with such a notion, since such fluid uptake is probably largely extracellular, and when the medium is made hypotonic, to favor axonal water gain, then scattered light intensity decreases even in an extended beam, in qualitative agreement with the hypothesis.

In addition, however, hypotonicity probably also spreads axons and axon bundles and increases whole nerve diameter. Since the evidence shows that these latter changes increase the light scattered, finding a net scattering decrease in hypotonic solution means that the actual decrease in light scattering is greater than observed, since it also compensates for a simultaneous increase due to spread.

This is important, because, to explain the scattering decrease with activity, Hill ('50) compared scattering changes caused by activity with scattering changes produced by hypotonicity. Probable water entry due to known hypotonicity was calculated and the accompanying change in scattering noted. It was then assumed that the same scattering change due to activity in seawater would mean the same amount of water had entered. Next an attempt was made to account for this calculated water entry in terms of ion movements occurring with activity, and it was found that a large inward transfer of NaCl had to be postulated. This was not found to occur (Keynes and Lewis, '51). It is in this context that the experiments reported here have bearing, since they indicate that the net inward transfer of NaCl thought necessary was probably too large. Because of the double effects of hypotonicity, the estimate of water entry during activity was probably excessive, and hence not such a large inward transfer of NaCl would have to be postulated. How much of the NaCl transfer would turn out to be unnecessary is difficult to assess, since the results might be different with Carcinus nerves from what they are with frog nerves. At any rate, the present findings do not contradict the hypothesis, and, in addition, suggest a way in which it may be made more tenable.

SUMMARY AND CONCLUSIONS

1. The light scattering change produced by activity in frog nerve is described, and a degree of reproducibility is demonstrated.

2. The phenomenon is shown not to be highly temperature sensitive.

3. The phenomenon appears not to be influenced by the white light so far used for its detection.

4. The straight, fully immersed nerve produces a larger scattering change with activity than does the bent, partially immersed nerve.

5. Neither stretch of the nerve nor dilution of the medium has reversed the direction of the frog nerve response as does occur with Carcinus nerves.

6. Desheathing increases the optical response though it does not decrease the total light scattered.

7. The importance of illumination area is demonstrated: (a) In an incident beam larger than the nerve, weight gain in Ringer solution is associated with an increase in scattered light. (b) In an incident beam smaller than the nerve weight gain in Ringer solution is associated with a decrease in scattered light. (c) In an incident beam larger than the nerve immersion in hypotonic solution is associated with a decrease in scattered light.

8. In an incident beam smaller than the nerve the optical response with activity is greater than in an incident beam larger than the nerve.

9. The significance of these findings for interpretation of the phenomenon is discussed.

LITERATURE CITED

Bryant, S. H., and J. M. Tobias 1952 Changes in light scattering accompanying activity in nerve. J. Cell. Comp. Physiol., *40:* 199–220.

———— 1955 Optical and mechanical concomitants of activity in Carcinus nerve. I. Effect of sodium azide on the optical response. II. Shortening of the nerve with activity. J. Cell. Comp. Physiol., *46:* 71.

Hill, D. K. 1950 The effect of stimulation on the opacity of a crustacean nerve trunk and its relation to fiber diameter. J. Physiol., *111:* 283–303.

———— 1950a The volume change resulting from stimulation of a giant nerve fiber. J. Physiol., *111:* 304–327.

Hill, D. K., and R. D. Keynes 1949 Opacity changes in stimulated nerve. J. Physiol., *108:* 278–281.

Keynes, R. D., and P. R. Lewis 1951 The sodium and potassium content of cephalopod nerve fibers. J. Physiol., *114:* 151–182.

Shanes, A. M. 1953 Effects of sheath removal on bullfrog nerve. J. Cell. Comp. Physiol., *41:* 305–312.

Tobias, J. M., and S. H. Bryant 1955 An isolated giant axon preparation from the lobster nerve cord. Dissection, physical structure, transsurface potentials and microinjection. J. Cell. Comp. Physiol. (in press).

OPTICAL AND MECHANICAL CONCOMITANTS OF ACTIVITY IN CARCINUS NERVE

I. EFFECT OF SODIUM AZIDE ON THE OPTICAL RESPONSE

II. SHORTENING OF THE NERVE WITH ACTIVITY

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Effect of sodium azide on the optical response

The rationale of this first set of experiments can be stated relatively easily. It has been demonstrated that nerve trunks (frog, Carcinus, spider crab, squid) (also lobster and Limulus leg and optic nerves, Bryant and Tobias, '54) transiently and more or less reversibly change their light scattering properties with activity. Such a change may be (1) a pure decrease in scattering, the response characteristically produced by a lax nerve in normal or hypertonic media, (2) an increase in scattering, as is produced by activity in a hypotonic medium or while maintained long axis stretch is applied to the nerve, or (3) a diphasic response in which the increased scattering phase always precedes one of decreased scattering (Hill, '50a; Bryant and Tobias, '52; Shaw and Tobias, '55). In addition, it has been reported that stimulation of Sepia giant axons produces shrinkage followed by swelling (Hill, '50b).

It has been suggested that (a) the decreased scattering response is associated with axonal water uptake and swelling resulting from sodium entry, and (b) that the increased scat-

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tering response may be caused by axonal shrinkage attendant either upon exchange of hydrated potassium for non-hydrated sodium, or upon water extrusion due to elevated intraaxonal pressure. Either of these mechanisms would produce some shrinkage though simple ion exchange seems inadequate. Since no scattering change has yet been reported for single axons, and though, until now (see Part II) no dimensional change has been reported for nerve trunks, the suggestion of a causal relationship between the two sorts of changes has been entirely inferential. In addition, objections have been raised concerning the hypothesis (Keynes and Lewis, '51), and certain technical matters which bear on quantitation of the responses, and thereby on interpretation, have been explored (Shaw and Tobias, '55).

Recently it has been reported that 3.0 mM sodium azide markedly reduces sodium efflux from Loligo or Sepia axons without significantly interfering with excitability or transsurface potentials. Sodium entry is said to be less affected though it apparently is somewhat reduced (Hodgkin and Keynes, '53).

If one tentatively assumes a similar action for other forms, then, if the hypothesis is correct, treatment with azide should, by slowing sodium egress, prolong and perhaps increase the magnitude of the decreased scattering response. Such coincidence of effects would not prove a causal relationship, but would be compatible with it.

Concerning the increased scattering response: If it is due to water extrusion then, for the same reasons as given above, azide might diminish the response both in magnitude and duration.

Therefore the effect of azide on the three types of scattering response has been investigated.

METHODS

In each experiment three optical responses were first recorded at 5 minute intervals, with the nerve in normal sea water, to provide reference values. The chamber was then

refilled with azide sea water, and responses were again recorded at 5 minute intervals until the nerve was nearly or completely inexcitable. Control nerves were treated in the same way but without azide. Stimuli were adjusted to produce maximal electrical activity, and were delivered at the rate of 30 shocks per second for 10–15 second periods.

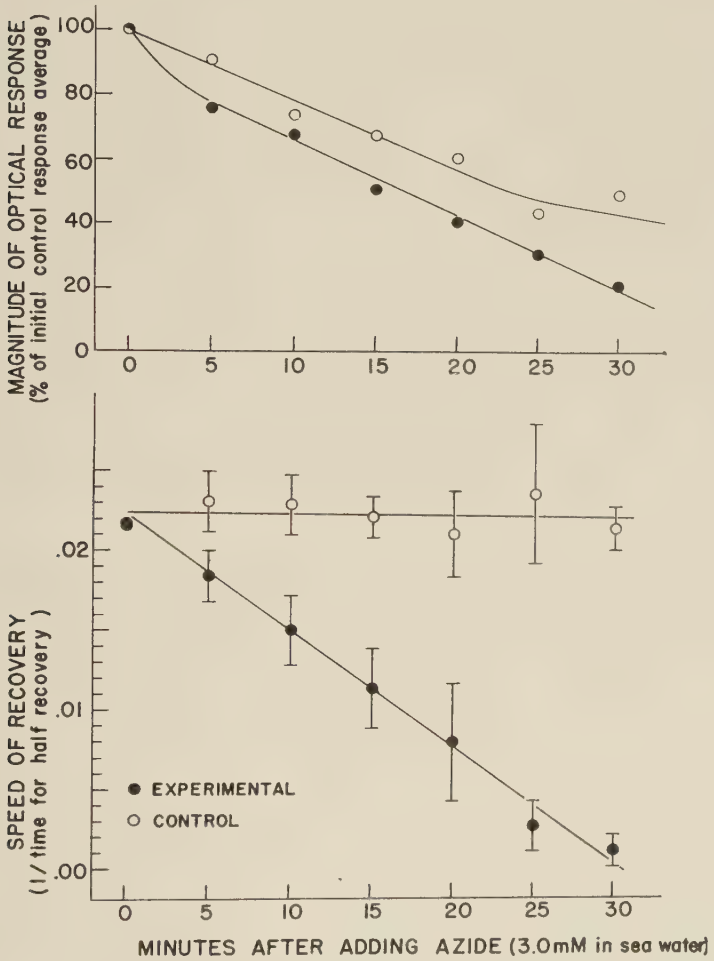


Fig. 1 Effect of sodium azide on magnitude of and recovery from the decreased scattering response of Carcinus nerve.

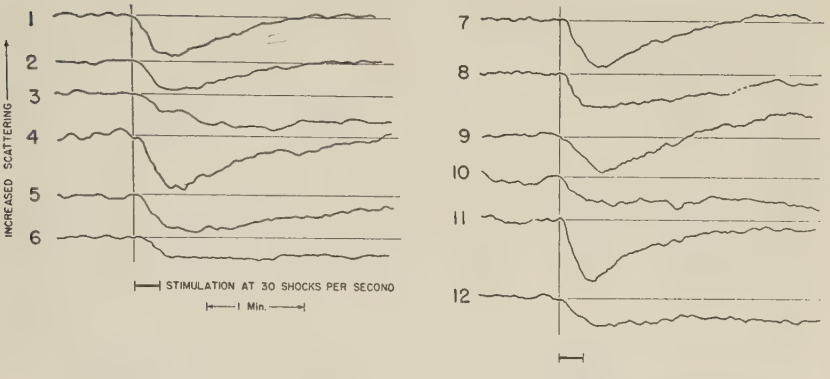
Decreased scattering responses were obtained by having the nerve lax in the chamber. Increased scattering responses were obtained by applying maintained stretch to the nerve (Bryant and Tobias, '52).

Usually the optical response with activity is superimposed on a basal drift in scattered light intensity. In estimating the magnitude and duration of responses such basal drift was corrected for, using the drift rate in the minute preceding stimulation as its best measure.

The quantities graphed in figures 1 and 4 require difinition: Optical response magnitude refers to the maximum increase or decrease in scattered light intensity occurring after the start of stimulation. By recovery is meant return of the scattered light intensity toward or to the resting level. Half recovery time was used in the calculations because of greater stability over the shorter interval, and was measured in seconds from the start of stimulation. When no recovery occurred during the 5 minute interval between stimulations then the recovery time was considered to be infinitely long. Because of the difficulty in handling infinity in statistical evaluation the reciprocal of the half recovery time was used, this quantity being called speed of recovery.

RESULTS

Decreased scattering response. With the nerve lax in normal sea water, so as to evoke a scattering decrease with activity, gradually increasing conduction failure became complete at anywhere from 20–60 minutes. There is reason to believe that the submerged part of the nerve survives longer. Addition of 3.0 mM azide hastened failure slightly if at all, and with or without azide the small, slower conducting fibers failed first. The optical response usually disappeared concurrently with small fiber failure, and occasionally became undetectable even though some of the larger fibers continued to conduct. After the nerve failed it was usually possible to get some recovery, electrical and optical, by washing with sea water.



RECORD	NERVE	DESCRIPTION
1	12C	CONTROL
2	"	14 MIN IN AZIDE
3	"	25 MIN. IN AZIDE
4	14C	CONTROL
5	"	10 MIN. IN AZIDE
6	"	35 MIN. IN AZIDE

RECORD	NERVE	DESCRIPTION
7	17C	CONTROL
8	"	7 MIN IN AZIDE
9	18C	CONTROL
10	"	20 MIN. IN AZIDE
11	63C	CONTROL
12	"	26 MIN IN AZIDE

NOTE: All the above responses are tracings from uncorrected original records. Controls are in normal sea water. All others are in 3.0 mM sodium azide containing sea water.

Fig. 2 Effect of sodium azide on the pure decreased scattering response of Carcinus nerve.

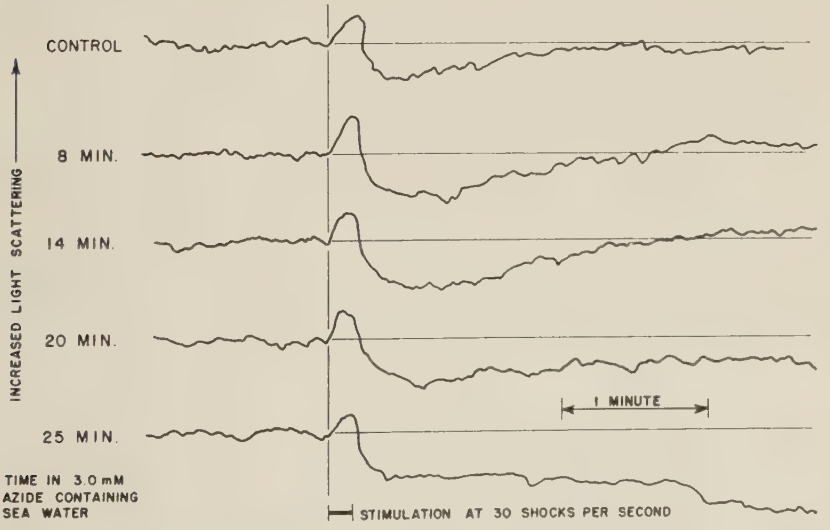


Fig. 3 Effect of sodium azide on diphasic scattering response of Carcinus nerve.

The records were examined to see if azide affected either the magnitude of or recovery from, the scattering decrease. Figure 1 shows that, as time passed, the response became smaller in three control nerves at about the same rate as in 6 azide treated nerves. Azide may have accelerated failure of the response slightly, but the data do not prove this. Recovery from the scattering decrease, however, seems definitely to have been progressively slowed by the azide. Figures 1, 2 and 3 show this to have been the case whether the optical change was a pure scattering decrease or was the secondary scattering decrease of the diphasic response. After sufficient time in azide there was no recovery at all.

This demonstration that azide has only a very small if any effect on the magnitude of the scattering decrease as contrasted to its large effect on recovery is of interest in terms of interpretation. Genesis of the scattering decrease is less sensitive to azide interference with metabolism than is recovery from it. It may be justifiable to paraphrase this in terms of passive and active responses.

Increased scattering response. The data concerning azide action on the increased scattering response seem to lead to clear conclusions, but certain considerations render interpretation less satisfying.

Figure 4 shows that, as before, response magnitude fell off at about the same rate in control as in azide treated nerves. In addition, the lower curves, from pooled data on all increased scattering responses, indicate that now, in contrast to the results with decreased scattering responses, azide did not affect recovery. The results, taken at face value, therefore mean that the increased scattering response differs from the scattering decrease in that recovery from the latter is sensitive to azide whereas recovery from the former is not.

This would be extremely interesting, and may be true, but there are serious reservations: Clearly, measurement of recovery time can yield interpretable data only if the recovery rate is not interfered with by some superimposed phenomenon. For example, when the response is a pure scattering increase

recovery is characterized by gradual return to the baseline. If the response is diphasic, however, then the increased scattering phase often returns to the baseline with a steeper slope. Therefore one must consider that onset of the scattering decrease, in diphasic responses, may encroach on the underlying, more gradual, true recovery from the preceding scattering increase, obscuring that slower recovery and making it appear to be more rapid than it really is. Since in the

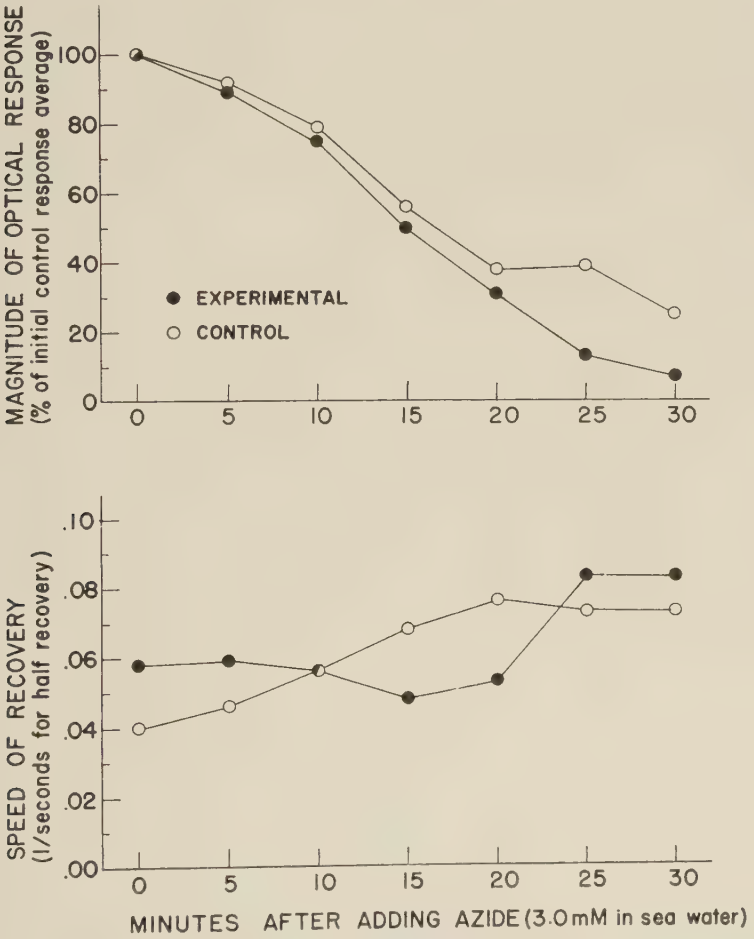


Fig. 4 Effect of sodium azide on magnitude of and recovery from the increased scattering response of Carcinus nerve.

actual experiments the stretched nerves did adapt with time and gradually shifted their responses from initially pure scattering increases to diphasic responses, this might have been the reason why, as time passed, there appeared to be an overall increase in recovery speed with both control and experimental nerves as shown in the lower curves of figure 4.

Because of such a possibility the pure increased scattering responses were separated from the diphasic ones. Though more experiments were done, adaptation interfered with production of pure scattering increases and there were relatively few to draw upon. However, from 11 such responses given by 5 control nerves in normal sea water complete recovery occurred in all cases. After azide only 5 pure increased scattering responses were seen, these being produced by three nerves, but from three of these there was no recovery, and recovery from one was questionable.

Such considerations tend to support the notion that recovery from the increased scattering response is also slowed by azide, but this may be obscured by the onset of the scattering decrease when the whole optical change is diphasic. If such interaction were additive then the scattering decrease part of the diphasic response should be smaller and slower in developing after azide. The data show no such effects.

It would appear then that 3.0 mM azide does not significantly affect magnitude of the scattering responses. It does prolong recovery from the scattering decrease type of response. Its effect on recovery from the scattering increase is difficult to evaluate.

Shortening of the nerve with activity

Whether or not nerve changes length with activity is interesting for several reasons: (1) At the level of cellular excitation physiology the findings of such a change would suggest further experiments which might help clarify the molecular structural phenomena underlying the increase in transsurface conductance with activity, perhaps the core physicochemical

event in excitation. (2) Structural modifications of nerve cell somata or processes are of interest in terms of speculations about mechanisms underlying changes in synaptic resistance with use, learning, etc. (Tobias, '52; Eccles, '52). (3) Mechanical movements of nerve could confuse the studies on light scattering.

For these reasons a length change with activity was sought for and subsequently found to occur in walking leg nerves of Carcinus maenas. There is evidence that it also occurs with lobster nerve. It has not yet been seen using frog nerve.

The methods used allowed nearly simultaneous recordings of length and light scattering changes from regions of the nerve within a few millimeters of each other. Thus it was possible to carry out certain experiments bearing on possible relationships between the two phenomena.

METHODS

Figure 5 diagrams the apparatus. A bit of platinum foil, *ca.* 1–2 mg, was hung as a reflector on the middle of a submerged nerve segment in a chamber like that used before. This produced a sag of 0.2–0.7 mm as measured with a cathetometer. The chamber was then installed on the photoelectric device used in the detection of scattering changes, and by moving it to one side or the other the light beam could be made to fall either on the nerve or on the rider. Then a change in light scattering by the nerve or in position of the rider would increase or decrease the amount of light reaching the detector photocell through a 1×6 mm slit.

The photocurrent change resulting from reflector movement was translated into vertical displacement as follows: Prior positioning of the lens, rider and slit yielded a combination giving maximum photocurrent. Moving the lens up or down then sharply decreased the output. For standardization that slope of the lens-position-photocurrent curve relating a decrease in photocurrent to downward lens movement was used. The region where photocurrent was half maximal proved a satisfactory zero point. The light output

at this setting was balanced by adjusting the amount of light received directly from the source by an opposing photocell. When balanced, movement down or up of the lens, and by corollary, up or down of the reflector, was evidenced as a positive or negative voltage change across the photocell load resistor. The slope of the photocurrent-lens movement curve was determined by moving the lens with a screw through

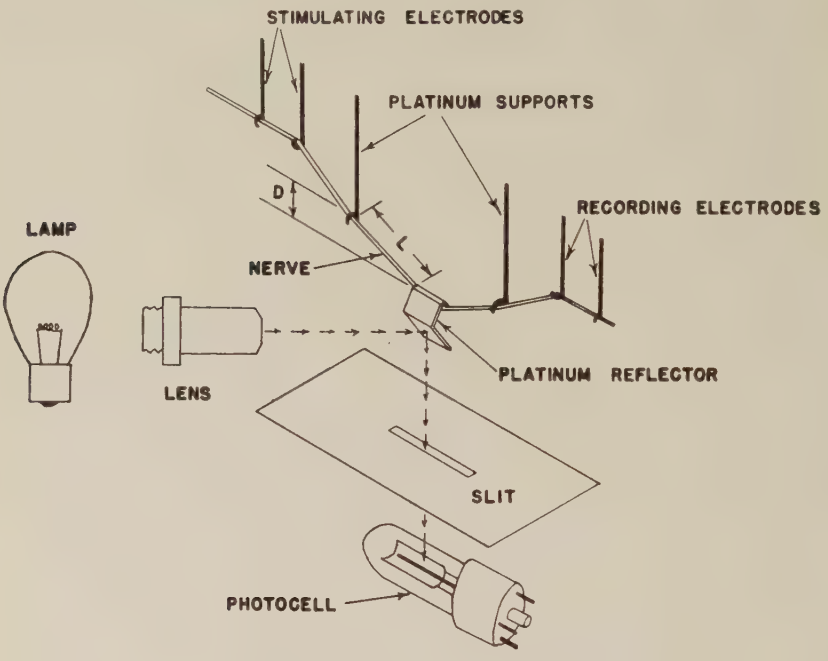


Fig. 5 Optical scheme for recording length change in nerve.

distances measured with a dial gage, reading estimated to 0.0001 inches, sufficiently to produce reasonable responses on a meter measuring voltage across the photocell load resistor. Vertical displacement of the lens by 25 μ resulted in development of about ± 0.25 volts across a 10.7 megohm resistor. Output was a linear function of movement within ± 30% over a range of ± 75 μ. After amplification, changes in the balanced output were recorded by an ink-writing milliammeter.

Major advantages of this system were several: (1) Sensitivity was high. The changes observed ranged from 0.1–3.0 μ, full scale deflection being equivalent to 2 μ. The apparatus was, however, stable at still higher sensitivities. (2) Using a short segment of nerve reduced low frequency vibration. Higher frequency vibrations were damped by the medium. (3) It was relatively easy to observe scattering and dimensional changes in the same nerve at regions and at times close together.

Filtered sea water was used throughout, its osmotic pressure being altered either by dilution with distilled water or by adding sucrose or NaCl.

Assumptions and controls referring to physical arrangement. 1. It was assumed that rider movement was produced only by changes in the central nerve segment, and that friction between the nerve and the central supports prevented the rest of the nerve from contributing to the change. As confirmation, large, directly visible changes induced at the stimulating electrodes by large currents produced no detectable rider movement.

2. Moving the lens with the reflector stationary was assumed to produce the same directional change in photocurrent as moving the reflector with the lens stationary. Direct mechanical trials showed this assumption to be correct.

3. The sagging segment of nerve, because of loading with the rider, was assumed to approximate a triangle rather than a catenary. Comparison with a straight edge verified this assumption.

4. Since the recorded changes were small compared to the dimensions of the elements involved, a linear system was assumed throughout. By way of confirmation, reflector movements when using nerve segments with much different sags calculated to about the same magnitude per unit length.

Being satisfied that the photoelectric response did measure a vertical shift of the rider, this vertical displacement was then translated into length change in the nerve segment as follows: The total sag, D, was measured by a cathetometer.

It was usually of the order of 0.5 mm, and since the cathetometer could be read to 0.1 mm the error of this measurement was of the order of 20%.

Now, if 2N be the constant distance between the central supports and L be one-half the actual length of nerve between the supports then

$$L = \sqrt{N^2 + D^2}$$

If the overall changes are small, and if D is small compared to L then it can be shown that

$$\frac{\Delta L}{L} \cong \frac{D}{N^2} \Delta D$$

The change in D, Δ D, was measured by comparing the change in light intensity with the calibration curve.

Though sensitive the method has been crude as used, it being estimated that the error may have been $\pm$ 100% at worst and about $\pm$ 30% on the average. More refined experiments could be done if (a) the amount of sag were more accurately measured, and if (b) the calibration were made more precise.

RESULTS

For reasons to be specified, elevation of the rider is considered to mean shortening of the nerve segment supporting it. An alternate, valid interpretation may have been overlooked, but the controls, which will be described, indicate that real shortening does occur. Anticipating such justification, the results will now be described in terms of "length change."

Length change per se. Ninety-six stimulations of 26 nerves, at osmotic pressures from 70% to 120% that of normal sea water, all produced shortening. In normal sea water, 35 stimulations of 19 nerves produced an average shortening of 0.03 μ per millimeter per 1000 impulses (extrapolated) (table 1). The resting drift during 97 pre-stimulation periods, using 26 nerves, randomly distributed among the media, was in the direction of lengthening in 82, shortening in 13 (10 of these with one nerve) and questionable in two. There was no latency greater than the instrumental lag of 0.2 sec. The

shortening response exhibited a definite threshold correlated with electrical responses. As stimulation intensity was increased both the electrical and shortening responses increased to a maximum plateau (fig. 6).

TABLE 1

Correlation of optical responses and shortening by active Carcinus nerve

(All nerves maximally stimulated 30 times per second)

NORMAL SEAWATER		HYPOTONIC SEAWATER			20% HYPERTONIC SEAWATER	
$\frac{\Delta L}{L}$	$\frac{\Delta I}{I} \times 100$	per cent tonicity	$\frac{\Delta L}{L}$	$\frac{\Delta I}{I} \times 100$	$\frac{\Delta L}{L}$	$\frac{\Delta I}{I} \times 100$
.10						
.11						
.038	— 1.12					
		85	.028	— 1.05		
		80	.067	— 0.58		
		80	.018	+ 0.13		
		90	.11	— 1.02		
		85	.012	diphasic		
		85	.035	— 1.72		
		85	.011	— 0.69		
.019						
.006	— 0.75					
.042						
.046	— 1.13					
.013	— 1.88	85	.022	— 1.65		
.022	— 1.20	70	.083	+ 10.5		
.003	— 0.64					
.028	— 2.00	75	.069	+ 11.4		
.049	— 0.84	75	.203	+ 40.8		
.045	— 4.06	75	.090	+ 20.1		
.038	— 2.98				.005	— 1.08
.074	— 1.82	75	.191	+ 16.6	.029	— 0.71
.006	— 1.38					
.005	— 0.48					
.006						
.008						
means						
.034	— 1.56					

$\Delta L/L$ means decrease in length per 1000 impulse burst in microns per millimeter of nerve.

$\Delta I/I$ means per cent change in 90° scattered light per 1000 impulse burst.

Due to drift and fluctuations only 41 of the 97 responses were analyzed for recovery speed. Of these, 33 showed complete or partial recovery, 6 showed none and two were questionable. Half recovery time averaged 36 seconds (range 26–57) (33 stimulations of 15 nerves). The full recovery time, measured in 7 nerves exhibiting 13 responses from which complete recovery occurred, averaged 77 seconds. There was no significant effect of osmotic pressure on recovery time.

Certain additional controls now became necessary for interpretation. (1) That spread of the stimulating current was

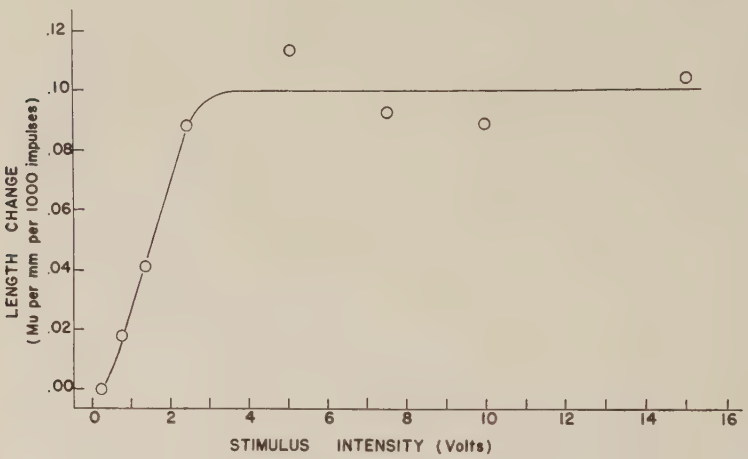


Fig. 6 Length change of Carcinus nerve as a function of stimulus intensity.

not causing the length changes was shown by the same sort of procedure as had been used for the same problem in connection with scattering changes. Since effects due to current spread would probably not exhibit a threshold or plateau coincident with the threshold and plateau of electrical responses in an excitation-response curve, the shortening magnitude was determined as a function of stimulus voltage for constant, 1 ms. pulses delivered at a rate of 30 per second (fig. 6). The curve supports the notion that the length change is a function of nerve activity and not of the stimulus. In addition, visual monitoring of the nerve impulses showed

that, as with the scattering response, the length change was primarily related to activity in the smaller fibers. Further, when the nerve became inexcitable after KCl or procaine treatment or simply because of deterioration, then delivery, over a comparable time, of as much as 100 times as many coulombs as usual failed to cause shortening. Development of the length change therefore required the presence of propagated electrical activity and was quantitatively related to it. (2) Twisting of the nerve and reflector could occur along with or instead of vertical movement. The changes observed have, however, always been in the same direction independent of randomly mounting the nerve. (See also item 4.) (3) The instruments used might have forced the nerve to react in a constant fashion regardless of its inherent tendency. As shown in item 4, however, shortening occurred even when the geometry was radically changed. (4) Finally, and perhaps most important, the question arose as to whether elevation of the reflector really meant shortening of the nerve. There was, for example, the possibility that an increase in nerve radius, without vertical shift of the center, could elevate the reflector and give the impression of shortening. Three types of evidence make it quite certain that shortening did occur: In 5 experiments the nerve was hung vertically, in a modified chamber, with a reflector attached to its lower end. Stimulation at the top elevated the reflector. While swelling could have been responsible, there can be no doubt that shortening did occur. Ideally, however, one would prefer to prove shortening in the same experimental situation as used for the majority of experiments. Therefore, in 4 trials the nerve was mounted as shown in figure 5, but extended only to midway between the Pt supports, at which point it was joined to a length of thread which was run along the rest of the Pt wires just as the nerve normally was. Then the reflector was hung on the thread. Now the nerve was stimulated and again the reflector rose. After addition of KCl no such responses were seen. It is difficult to see how such could have been produced without shortening of the

nerve. Finally, by good fortune, the reflector in one experiment was held by surface tension of the medium in such a way as to produce an "upward sag" of the nerve. Activity in this case caused the rider to descend, again indicating shortening of the nerve. From the evidence one may conclude that Carcinus nerves, as used, did shorten. Therefore one can discuss experiments done in an attempt to detect correlation between shortening and the scattering changes.

Correlating experiments on shortening and scattering responses. Alternate recordings of light scattering and dimensional responses were made, under different osmotic conditions, to find if any directional or other quantitative correlation exists between the two responses. As mentioned, either long axis tension or osmotic pressure may be manipulated to evoke one or another type of scattering change with activity. Osmotic pressure was used here since changing it does not involve mechanical rearrangements as gross as those accompanying stretch. Hypotonicity causes the nerve to increase its light scattering with activity. However, as also with stretch, the nerve adapts, begins to produce diphasic responses, and sooner or later reverts to producing pure decreased scattering responses. For this reason it was difficult to obtain the desired combinations of responses on demand and in predetermined order. However, enough experiments were done so that it is possible to draw certain conclusions.

Table 2 shows that, (1) osmotic pressure did not influence the direction of the length change with activity, it being always a shortening, and (2) this was the case (see particularly the starred boxes in table 2) whether the length change was bracketed in time by increased scattering, diphasic or decreased scattering responses.

The data in table 1 were selected to give the most representative quantitative data for the various responses of each nerve under the conditions indicated. The values paired are those considered the have been the most probably concurrent ones. It will be seen that when hypotonicity caused the nerve

TABLE 2

Comparison of directions of scattering and length changes in active Carcinus nerve

os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL
100		S	100		S	75	+		100	—		100	±		85		S	100	±		100		S	100	—	
	—				S		+				S		—				S		—			—			S
70	+			—				S	75	+				S		±			—			S	120	—	
	+		75	+				S			S	120	—			±				S	85		S		—	S
		S			S		+			+				S			S			S		—				?
		S		—			+				S			S			S		—	S	70		S			?
				—		100	—			+		100	±			±			—			±		?		
							—			—				S		+			±				S	100	—	
								S			S	75	—			±			—			—			S
								S			S			S			S					—			S
							—					100	—				S						S			
							—							S									S			
								S						S												
							⚹				S					⚹			⚹							

os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL	os.p.	ΔI	ΔL
85	±		80		S	80		S	90		S	85		S	85		S	100		S	100	—		100		?
	—				S		+				S			S			S			?		—				S
	—			—						—			—			—			—			S			?
		S		—						—			—			—				?		S		—		
		S		—									—				S			S				—		
		S			S									S			S			?						S
														S			S									S
													—													

Notes: (1) os.p. means osmotic pressure of the medium as % of osmotic pressure of normal seawater. (2) ΔI refers to change in light scattering; + means increase, - means decrease, and ± indicates a diphasic response. (3) ΔL refers to change in nerve length, S meaning shortening.

Temporally successive observations are arranged vertically, the earliest at the tops of the columns. Each box contains data for a single nerve.

to increase its light scattering with activity the response was much greater than the scattering decrease given by the same nerve in normal sea water, i.e. hypotonicity both reversed and amplified the response. Increased tension has the same amplifying effect (Bryant and Tobias, '52). The concurrent length change, even though its direction was unchanged, tended also to be amplified by hypotonicity.

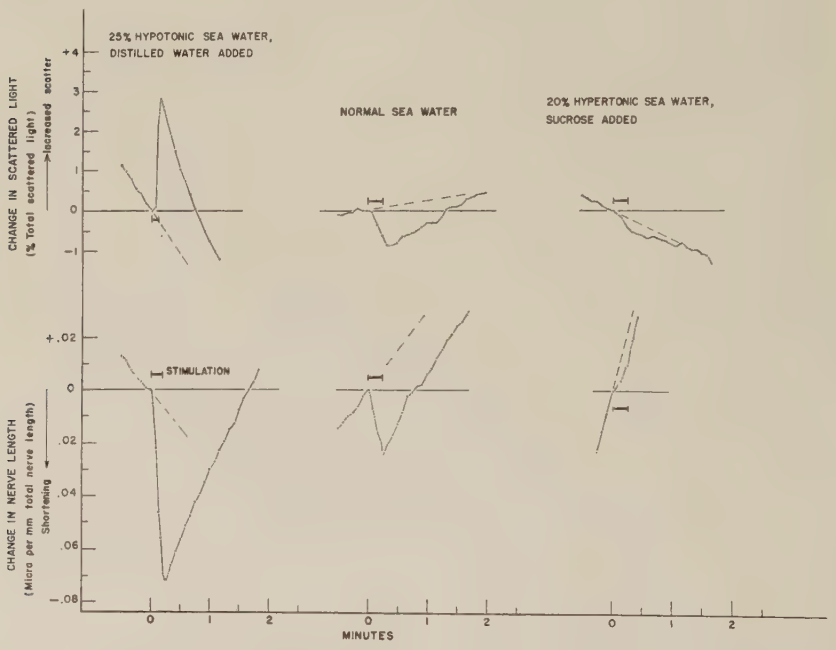


Fig. 7 Length and scattering changes in a single Carcinus nerve immersed in different media.

The data are exemplified by the curves of figure 7 taken from the record of a single nerve with which it was possible to observe both length and scattering changes in normal, hypertonic and hypotonic sea water.

DISCUSSION

Azide experiments. The experiments of Part I are of interest primarily because they demonstrate differential sus-

ceptibility of the several parts of the Carcinus nerve optical response to azide.

Azide has no effect on the size of the increased scattering response and little if any on the size of the decreased scattering response. Pertinent correlates may be the small effects of azide on axonal spike height and sodium entry (Hodgkin and Keynes, '53). On the other hand, recovery from the scattering decrease is definitely prolonged, and recovery from the scattering increase may also be prolonged. Again possible correlates exist: It is known that azide can suppress the increase in oxygen consumption normally following activity without blocking the activity itself (Brink et al., '52). If this usual oxygen consumption increment represents energy made available for long term recovery, then, when it is suppressed recovery deficits such as delayed sodium extrusion and delayed optical recovery might be expected. Put more generally, it is as if the optical changes are passively generated in the same sense that generation of the spike and sodium entry are immediately passive, and therefore none of these is markedly sensitive to azide. Long term recovery, however, requires metabolism and is therefore sensitive to azide, such interference showing up in optical response prolongation and in sodium retention. These findings bear out a point of view voiced elsewhere, namely that both genesis of and immediate recovery from the electrical change during activity are driven by pre-existing potentials and reflect electrokinetically induced structural changes which are intrinsically independent of metabolism (Tobias, '52a). The results are also consonant with, though they do not prove, the point of view that azide interference with metabolism prolongs the decreased scattering response by delaying sodium extrusion.

The insensitivity of response magnitude as compared to recovery is of interest for another reason. If optical recovery normally occurred coincidentally with the scattering change then delay or absence of recovery should increase response magnitude. The fact that it does not do so suggests that recovery may normally start only after stimulation is over.

If this is true and if optical recovery does depend on sodium extrusion then the findings suggest that sodium extrusion is held in abeyance during activity. In current parlance, it is as if the "sodium pump," or whatever it is that behaves like a sodium pump, is shut off during activity. An alternate possibility is that Na efflux is not suspended during activity, but rather accelerates only after activity terminates.

In addition to such ionic pump model possibilities, one must also consider that the scattering changes, even if water moves as has been suggested, may be due to structural changes in axonal colloids (Tobias, '52a) which are dependent upon azide sensitive metabolism for their structural state.

Finally, as also suggested before (Tobias, '52b), it is seen that azide is a tool which allows one to accumulate certain changes resulting from activity, which are normally so small and evanescent as to be detectable only with difficulty. Thus, whatever it is that underlies the scattering decrease with activity may be preserved and accumulated in time by azide just as one of its signs, the change in light scattering, is preserved.

Shortening experiments. Though ill-defined scattering changes seem to occur after bursts of higher frequency stimulation, no scattering change of the usual sort has yet been reported for single isolated axons, though it has been looked for (Bryant and Tobias, '54). Nor has any length change been reported for isolated axons, it also having been sought (Hill, '50b; Cragg, '51; Bryant and Tobias, '54). Shortening of frog axons in the region of a polarizing metallic anode (Tobias, '51) may be entirely unrelated to the presently reported phenomenon. At least the data warrant no correlation. Therefore one still does not know the precise microanatomical parts whose scattering properties change or whose structural modification results in shortening. There is much in nerve trunks besides axons. Concerning the matter of localization, however, it may be repeated that both the scattering and length changes, as observed so far, seem to depend on activity in the smaller fibers. Because of differences in surface-volume

ratio, the changes may be much smaller in the giant fibers, and, since all the single axon work along these lines has been done on giant fibers, this may be the reason why neither the scattering nor length changes have yet been seen.

Apropos mechanism: Shortening could be a change in form due to water uptake, or could result from active molecular reorganization, perhaps something like muscular contraction much attenuated. The data do not yet justify much speculation on this issue, though it is potentially the most interesting point raised, but the absolute magnitude of the change requires discussion.

Shortening, in normal sea water, was about 0.03 μ (300 Å.U.) per mm of nerve per 1000 impulses. This is equivalent to 0.3 Å.U. per mm per impulse, and since such a dimension is even smaller than atomic diameters one might wonder about its meaning. Four points merit consideration: (1) Expressing shortening as microns per mm per 1000 impulses is simply a convenience and is not meant to imply a linear relation among these quantities, no dose-response curve describing them as related functions being yet available. All data were obtained for an essentially fixed frequency. Therefore interpolation to the change per impulse is hazardous. (2) The observed shortening has been an overall change in a comparatively large piece of tissue. To shorten the nerve 0.3 Å. U. by amputation would, at constant radius, result in removing about 1×10^{-12} cc. In terms of a protein of molecular weight 100,000 this volume is equivalent to about 1×10^{7} molecules. Therefore, even so small a shortening as has been seen represents a volume, in the whole nerve cross section, which is large in terms of molecular dimensions. (3) Quantitatively adequate data for a precise calculation are not at hand, but approximation suggests that ion exchange induced water movement could account for less than 1% of the observed shortening. (4) The measured shortening may be smaller than the real shortening occurring in the elements actively concerned, since if the length change is due to shortening of certain structural units they may

shorten primarily with slippage inside a relatively inert mass. Therefore the figure given may represent only a fraction of the change at molecular levels.

Turning now to experiments in which both length and scattering changes were measured, it will be recalled that increased scattering has been tentatively associated with axonal shrinkage and water loss, decreased scattering with axonal swelling and water gain (Hill, '50a, '50b). In support, it has been shown that hypo- and hypertonicity do produce decreased and increased scattering respectively by both Carcinus and frog nerves. In addition, in a number of the present experiments small changes in osmotic pressure showed that hypotonicity produced transient shortening, whereas hypertonicity accelerated lengthening, larger tonicity changes producing larger responses. Therefore, in terms of the above hypothesis, one might expect the decreased scattering response, said to involve water uptake, to be accompanied by shortening, and the increased scattering response, said to involve water loss, to be accompanied by lengthening. No such correlation has been found. In all cases the activity response was in the direction of shortening, whether the concurrent optical change was an increase or a decrease in scattering. Nor has any diphasic length change been seen. The water movement hypothesis (Hill, '50a, '50b), therefore, does not provide a framework into which the present findings fit simply and obviously.

The invariance of the shortening response, taken together with the fact that hypotonicity (water gain) produces shortening and decreased scattering, suggests that the increased scattering response may be produced by a mechanism fundamentally different from that responsible for decreased scattering or shortening.

Certain positive correlations between the length and scattering changes also deserve attention: Both show roughly the same time span, and in both recovery is slower than onset. Both may also show overshoot. Secondly, though hypotonicity does not reverse the length change as it does the scattering

response, it does amplify both. Both responses seem to be associated primarily with activity in smaller fibers.

There remains the question of whether nerve shortening contributes to the light scattering responses. Shortening, by bringing additional opaque material into the beam could, *a priori,* either increase or decrease scattering. Whether an increase or a decrease would result would depend on whether the nerve were very opaque or not to begin with. Even without knowing on which part of the transmission curve one is operating, however, since hypertonicity is known to increase scattering by Carcinus nerves (Hill, '50a) the chances are that long axis shrinkage would tend in the direction of increased scattering. Therefore it would seem that the decreased scattering response could not under any circumstances be produced by such a mechanism, though it might be diminished thereby. If now, one also considers the problem quantitatively it turns out that the volume increase due to the observed long axis shrinkage would be less than 0.03% in normal seawater and less than 0.06% in hypotonic seawater. It does not seem likely that, even under the most favorable circumstances, such volume changes would be significant for either type of scattering change.

One other possibility remains. Shortening might change the mass of nerve in the incident beam due to changing the sag. Thus, if the light beam were focussed above the position on the nerve which would give maximal scattered light then shortening would, by reducing sag, elevate the nerve and bring more seattering material into the beam. With the light beam too low opposite results would be expected. When such arrangements have been tried the nerves always showed a scattering decrease with activity independently of whether the beam was focussed too high or too low.

The experiments therefore indicate that the scattering changes are not produced simply by positional or gross configurational changes due to shortening.

SUMMARY AND CONCLUSIONS

1. Sodium azide (3.0 mM) applied to Carcinus nerve has little if any effect on either the rates of generation or magnitudes of the optical responses with activity. It does, however, prolong, and finally prevents, recovery from the decreased scattering type of response. Its effect on recovery from the increased scattering response is not yet completely clear.

2. Implications of the above findings are discussed.

3. Carcinus and lobster leg nerves shorten as a result of electrically induced impulse propagation. Characteristics of this shortening are described for Carcinus nerves.

4. Shortening occurs whether the concurrent optical change is an increase or a decrease in scattering or is of the diphasic type. The matter of independence or interrelation of the phenomena is explored.

5. The scattering change is real and is not an artifact due simply to the shortening.

6. The question of the microanatomical locus of the elements responsible for the phenomena remains unanswered, but both the shortening and the scattering changes seem to depend primarily on activity of the smaller fibers. This suggests a correlation with axonal surface area but still does not localize the phenomena adequately.

7. Hypotheses relating to mechanism are discussed.

LITERATURE CITED

Bryant, S. H., and J. M. Tobias 1952 Changes in light scattering accompanying activity in nerve. J. Cell. and Comp. Physiol., *40:* 199–219.

———— 1954 Uupublished observations.

Cragg, B. G. 1951 The turgidity of giant axones. Jour. Physiol., *114:* 234–239.

Hill, D. K. 1950a The effect of stimulation on the opacity of a crustacean nerve and its relation to fiber diameter. J. Physiol., *111:* 283–303.

———— 1950b The volume change resulting from stimulation of a giant nerve fiber. J. Physiol., *111:* 304–327.

Hodgkin, A. L., and R. D. Keynes 1953 Metabolic inhibitors and sodium movements in giant axons. J. Physiol., 120 Proc.

Keynes, R. D., and P. R. Lewis 1951 The sodium and potassium content of cephalopod nerve fibers. J. Physiol., *114:* 151–182.

SHAW, S. N., AND J. M. TOBIAS 1955 On the optical change associated with activity in frog nerve. J. Cell. Comp. Physiol., *46:* 53.

TOBIAS, J. M. 1951 Qualitative observations on visible changes in single frog, squid and other axones subjected to electrical polarization. Implications for excitation and conduction. J. Cell. and Comp. Physiol., *37:* 91–105.

———— 1952a On ultrastructure and function in nerve. Nonelectrical physical aspects of excitation and conduction as deduced primarily from polarization experiments — in *Modern Trends in Physiology and Biochemistry,* ed. E.S.G. Barron. Academic Press, N. Y.

———— 1952b Some optically detectable consequences of activity in nerve. Cold. Spring Harb. Symp. Quant. Biol., *17:* 15–25.

ADDENDUM

It would be interesting, of course, if nerve shortening could be produced by the application of certain chemicals which might have something to do with the process of excitation. However, if care is taken to control pH and osmotic pressure, it has not yet been possible to produce a length change with 0.25% adenosinetriphosphate applied either to the living Carcinus nerve or to the nerve extracted with glycerol. Nor has tetraethylpyrophosphate or acetylcholine after tetraethylpyrophosphate produced any convincing length changes.

EFFECTS OF CENTRIFUGAL FORCES ON GROWTH AND FORM OF COLEOPTILE OF WHEAT[1]

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EIGHT FIGURES

THE PROBLEM

In many organisms, including man, an increase in stature is the most visible expression of growth. Material is lifted up and thus work is done against the pull of gravity, which is perhaps the most all-pervasive force in the biological environment. Even more strikingly than in the human being, the growth of a plant seedling is upward; we know that a response to gravity orients this growth and directs it through the mechanism of auxins produced in the growing tip.

But growth, says D'Arcy Thompson ('42), must be discussed as a vector of the dimensions of direction, magnitude and time. That gravity controls the direction of plant growth is known, but its effects on magnitude and time are little investigated. The problem may be stated: To what extent is the *form* and the *rate* of growth of the organism altered by gravity, against the downward pull of which it must lift itself? Put in experimental terms: How are height, diameter and cell size of a seedling affected? Is the rate of growth altered? And, ultimately, if the pull of gravity be doubled, will the plant do twice the work to raise the same mass to the same height?

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The coleoptile of wheat was selected as the experimental organism since it has a measurable response to gravity, a comparatively simple and distinct morphology and a short growth period. Furthermere, because they possess these qualifications, the seedlings of grasses have been widely studied as to their reactions to other environmental factors.

Centrifugation produces, by rapid rotation, forces in excess of the earth's gravitational field. Centrifugal forces many hundreds of times normal gravity are widely used to accelerate processes or accentuate tendencies normally produced by gravity. The possible effects of such forces on seedlings may be several. Protoplasmic elements, due to their differences in specific gravity, may be displaced or stratified and chemical processes altered. Transport may be altered from cell to cell and through vascular bundles where the weight of fluid columns is increased. More energy will be required to lift those substances (such as water) which travel upward, while descending substances will tend to move faster.

Structural adaptation may be necessary if mechanical failure is to be avoided. Since the effective weight of tissues increases as the centrifugal force acts, the burden of superposed structure upon the cells at the base of the seedling is many times greater than normal. Eventually, as Thompson observed, proportions must change, stronger materials must be used, or collapse will ensue; one of these three is inevitable.

Went ('54) makes the point that, "Because of the enormous advances made in biochemistry during the last 20 years, there is a tendency to overlook or discount the role of physical factors in plant growth and development in particular . . . Yet physical factors are of the greatest importance in experimental and theoretical work on growth and development . . ." Among these factors, altered gravitational forces have been investigated less than most of the environmental conditions affecting growth.

Few practical means exist for eliminating the effects of gravity in an experiment. In 1806 T. A. Knight devised a

turntable, which he called a *klinostat,* that would revolve a plant in a horizontal axis, thus neutralizing the pull of gravity. He reported that growth took place without reference to orientation toward the earth, and thus demonstrated the direction controlling action of gravity.

But gravity, thus neutralized, is not absent, and subsequent workers have found it easier to examine, by means of the centrifuge, forces increased beyond the approximately 980 cm per sec^2 due to the earth's attraction. Hertwig (1899) found that 3 to 6 times gravity slowed the development of frog eggs, and that 9 times gravity was lethal. Gray and Webb ('50) found that orientation and swimming patterns of tadpoles were disturbed after 10 days at 6 times gravity. In plants, Schecter ('35) altered the polarity of the alga, *Griffithsia,* at 150 times gravity.

More recent investigations of the effects of centrifugal force have turned to the use of high forces applied for short periods. Camara ('42) centrifuged wheat until chromosomal breaking occurred, and Saez ('41) produced stratification of the cell contents of seeds of *Lathyrum* at 3,000–6,000 times gravity. Pollen grain polarity was studied by Beams and King ('44) who found that reversal of polarity occurred in some, but not all, pollen grains subjected to 20,000 times gravity. Others, such as Conklin ('17, '31), Todd ('40) and E. N. Harvey ('31, '34) have worked on the effects of forces from 1,000 to 100,000 times gravity upon cell contents. Heilbrunn ('43) reports that Costello found that *Nereis* eggs withstood 200,000 for 10 minutes, and *Ascaris* eggs survived 400,000 times gravity for an hour. Hilbe ('42) produced polyploidy, giant cells and binucleate cells in germinating onion and rye seedlings at 50,000 times gravity and above. Similar effects were observed by Kostoff ('37).

It will be seen that little recent work has been done with low gravitational forces, and almost none with the effects of long-continued application of such forces. It is with low forces operating over considerable time that this paper is concerned.

MATERIAL AND METHODS

These experiments deal with the growth of the coleoptile of the germinating wheat seedling, *Triticum vulgare* L. Sanford winter variety, being used.

The growing axis of this plant is sheathed by the coleoptile, or third embryonic leaf (McCall, '34), which elongates just ahead of the true leaf primordia. In normal planting, this sheath protects the growing leaves for approximately two weeks, but this period is shortened to 4 or 5 days for the etiolated seedlings of the experiments. The coleoptile appears as a straight, translucent, greenish white shaft, elliptical in cross section, and of nearly constant diameter up to its cone-shaped tip.

In control plants the first true leaf, which grows up inside the coleoptile, pierces the tip of the latter at about 80 hours, at which time the plant is 30–35 mm tall. The coleoptile may grow 5 to 10 mm more after the appearance of the leaf before beginning its decline. That its growth is largely linear and thus easily measured, plus the fact that the coleoptile completes its growth curve in about 4 days, makes it an excellent subject for growth studies.

When uprooted, the seedlings are seen to possess a central primary root emerging from the embryonic tip of the seed in line with the coleoptile; on either side are one or two pairs of lateral seminal roots.

Histologically, the coleoptile displays a simple structure, being largely parenchymatous. A definite epidermis of a single layer of thicker walled, relatively longer cells surrounds the parenchyma. The latter is 6 to 9 cells thick except where thickened by two vascular bundles, one on either side in the larger diameter; these thickenings account for the normal elliptical cross section of the coleoptile. The single layer of inner lining cells is less differentiated than the outer layer.

In longitudinal section, the simple vascular relations of roots and coleoptile to endosperm and leaf becomes evident. The two vascular bundles of the coleoptile are derived from

the central vascular plate at the level of the first internode (Boyd and Avery, '36). This is the area of divergence of coleoptile from scutellum (specialized as an organ for absorbing food) and here is the short meristematic area of the coleoptile.

Figure 1 shows the outline of a seedling and its parts, as well as a diagram of measurements taken.

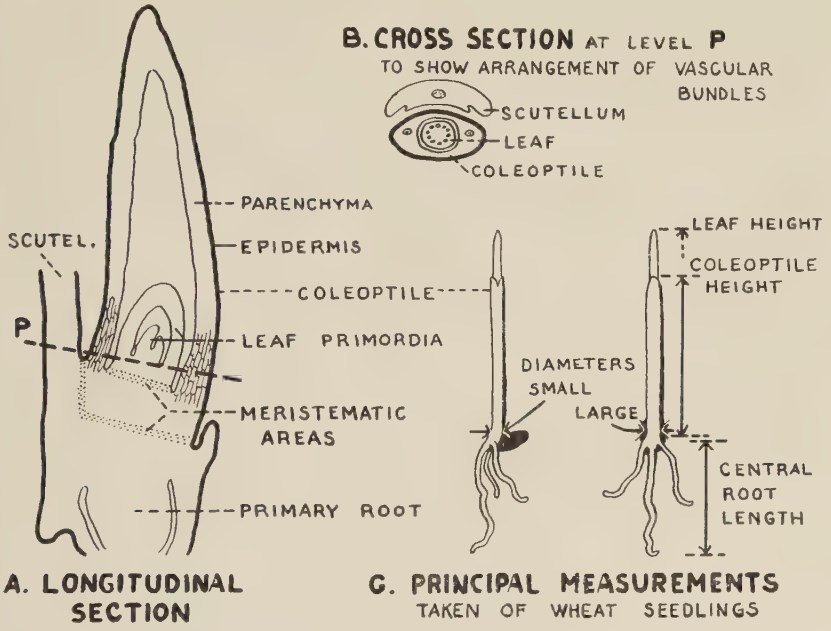


Fig. 1 Diagrammatic sections of a wheat seedling, and outline drawings showing principal measurements taken.

The wheat seeds were planted in centrifuge tubes on moist sand and grown in the dark. While control plants were grown in an incubator, experimental plants were centrifuged in an International Type 2 centrifuge at varying speeds during the period of growth of the coleoptile.

The procedure was as follows: selected seeds were sterilized for 30 seconds in 0.05% mercuric chloride and soaked for 30 minutes in water to initiate germination. The 50 ml

tubes were prepared with 25 gm of 20–30 mesh sterile Ottawa sand and 6–7 ml of boiled tap water; they were then centrifuged for 15 to 30 minutes to eliminate air spaces between sand grains. Four soaked seeds were arranged radially on the sand in each tube and the tube stoppered. Tubes to be subjected to supragravitational forces were placed in the centrifuge and control tubes put in an incubator in the same constant temperature room.

Throughout this discussion, the term supragravitational force or environment will be used to disignate conditions of increased acceleration in which

$$G = \frac{a}{g}$$

where a is centripetal acceleration and g is standard gravitational acceleration. Forces will be written: $25 \times G$, $50 \times G$, etc. From the formula for centripetal force, the horizontal acceleration of the centrifuge may be calculated:

$$G = \frac{(2 \text{ pi } N)^2 r}{980 \text{ cm per sec}^2}$$

where r is the radius in centimeters and N is the number of revolutions per second, while *980 cm per sec*^{2} was taken as the metric gravitational acceleration. Thus, to produce a force of $50 \times G$, for example:

$$50 \times G = \frac{(2 \times 3.1416 \times N)^2 \times 22 \text{ cm}}{980 \text{ cm per sec}^2}$$

where *22 cm* is the radius from the center of the centrifuge to seed level within tube and N is found to be 7.51 revolutions per second. Other values, evolved in like manner, were converted to revolutions per minute, and were as follows:

HORIZONTAL FORCE	RPM	HORIZONTAL FORCE	RPM
$10 \times G$	201.6	$150 \times G$	780.6
$25 \times G$	318.6	$200 \times G$	901.8
$50 \times G$	450.6	$500 \times G$	1425.0
$100 \times G$	637.8		

The vertical force of g ($1 \times G$) due to the earth's field was always present, of course, but its effect was quite small.

Thus, with a calculated horizontal force of 50 × G the actual resultant force placed upon the plant in the experiment was 50.01 × G. As the speed of the centrifuge varied with voltage changes caused by fluctuations in the electrical load on the local substation, it was not possible to maintain forces more accurately than plus or minus 5%.

All other factors known to alter rate of growth (temperature, light, nutrition, moisture and chemicals) except that of gravity were maintained as nearly constant as possible so that changes which were observed in rate of growth and final form attained might be attributed to alterations in the factor of gravity. There was no light in the room except for very brief periods while measurements were taken. The wheat received no nutriment other than that furnished by the starch of the endosperm itself and the minerals contained within the boiled tap water. By individually selecting seeds of similar size and fullness, the variation in amount of available nutrient was reduced to a minimum. Moisture was controlled by maintaining the level of water to the seed planting line and capping the tubes. The temperature of the room was controlled so that seedlings in centrifuge or incubator were maintained at 26° C. (± 1°C.).

The growing period was counted from the time of sterilization of seeds. After the first 24 hours, the seed level was marked on the tube, water added where necessary and the seeds covered with 5 gm of sand. All tubes were checked at 48 hours from zero hour, and at approximately 12 hour intervals during the rest of the growing period.

Growing coleoptiles were measured through a magnifying lens having an attached scale with vernier setting (Eberbach cathetometer) permitting measurements accurate to 0.1 mm of growth. At the end of the experimental period, the plants were gently uprooted and final measurements made of actual height, root length and diameters. Typical plants were selected for photographing and for making microscopic sections.

Serial longitudinal sections were made at 10–12 μ. The best sections were obtained following the use of Randolph's

variation of Craf's fixative (Randolph, '35) followed by dehydration in alcohol and normal butyl alcohol, and then slow infiltration of 56–58° paraffin for 15–18 hours. Sections were stained with Heidenhain's hematoxylin and counterstained with fast green.

The five most nearly mid-sagittal sections of each coleoptile were selected for parenchymal cell measurements. These were obtained by the use of an ocular micrometer in a compound microscope at 100 ×. In certain sections the size of meristematic cells was compared to that of parenchymal cells.

DATA

Growth may be evaluated by measurements of changing size. Coleoptile height, diameters and roots were measured, but since height shows the greatest change and is most directly influenced by gravity, other measurements usually have been related to height.

Height. The wheat coleoptile grows straight up from the end of its seed and thus its height is easily measured. The straight, vertical character of the coleoptile is not altered by speeds up to 100 × G. However, at 150 × G and even more at 500 × G, coleoptiles over 10 mm are remarkably curved. The curvature bears no constant relation to the position of the tubes in the centrifuge, or the direction of revolution. S-shaped curves are frequent and often the coleoptile lies flat on the substrate; it may even be broken in two by its own weight. It is, therefore, difficult to secure exact measurements on coleoptiles centrifuged at these higher speeds until they are uprooted at the end of the experimental period. Forces beyond 500 × G were not investigated because physical collapse prevents further adaptation to gravity on the part of the plant. Photographs (fig. 8) reveal characteristic outline of various experimental plants.

Height achieved at different hours, of course, reflects the rate of growth while final height when uprooted will reflect the total growth. Table 1 shows the height attained at various

hours of measurement for plants centrifuged continuously during the growing period of approximately 100 hours.

Rate of growth is reflected in figure 2. The height of the coleoptile increases in a typical sigmoid growth curve. The lower part of the accelerating phase of the curve is largely

TABLE 1

Height of coleoptiles (in millimeters) of plants centrifuged 0–96 hours

HOURS OF GROWTH	TYPE OF PLANT							
	CONTROL	Force applied						
	1 × G	10 × G	25 × G	50 × G	110 × G	150 × G	200 × G	500 × G
29	4.47 ± 1.79 (63)[1]							
48	9.26 ± 2.12 (135)	9.38 ± 1.82 (81)	9.58 ± 2.36 (49)	7.74 ± 1.48 (67)	7.97 ± 1.91 (77)	7.56 ± 2.12 (48)		
55	14.37 ± 2.86 (577)	14.94 ± 2.33 (129)	12.36 ± 3.85 (128)	12.39 ± 2.31 (118)	11.34 ± 2.96 (85)	11.70 ± 3.10 (51)	9.74 ± 1.77 (9)	
60	18.76 ± 3.80 (716)	20.03 ± 2.59 (129)	16.41 ± 3.54 (177)	16.67 ± 2.82 (140)	15.12 ± 3.05 (85)	13.23 ± 4.34 (71)	12.11 ± 2.79 (9)	
65	23.92 ± 4.00 (808)	25.03 ± 3.79 (141)	20.79 ± 3.67 (186)	20.74 ± 3.44 (148)	19.53 ± 3.35 (85)	15.14 ± 5.12 (85)	14.54 ± 1.85 (9)	9.00 ± 2.51 (49)
72	31.14 ± 4.70 (949)	32.00 ± 3.90 (141)	27.19 ± 3.93 (194)	26.33 ± 3.55 (177)	24.59 ± 3.26 (85)	18.22 ± 5.81 (94)	16.91 ± 4.34 (21)	10.80 ± 2.51 (49)
80	37.71 ± 4.32 (646)	36.52 ± 3.61 (142)	32.80 ± 2.75 (116)	31.91 ± 3.48 (181)	29.11 ± 3.24 (85)	22.18 ± 5.91 (102)	20.36 ± 4.57 (21)	13.10 ± 2.47 (49)
96	40.46 ± 4.20 (658)	38.92 ± 3.90 (138)	37.26 ± 5.58 (144)	36.63 ± 5.28 (181)	31.98 ± 3.10 (85)	27.03 ± 4.38 (103)	23.10 ± 3.99 (21)	17.57 ± 2.47 (49)

[1] Number of plants in parentheses.

concealed by the substrate. The linear phase of the control plants begins at about 48 hours (9.2 mm) after which growth is about 1 mm per hour. After the first true leaf emerges from the tip of the coleoptile at about 80 hours, the growth rate of the latter declines. Little further growth takes place after 96 hours, when the average height is 40.46 mm.

Seedlings subjected to centrifugation follow a similar sigmoid curve, but with altered height and slope. Plants subjected to a force of $10 \times G$ for the entire growing period show a slight but significantly accelerated growth rate up to 72 hours, after which they level off and do not quite attain the final height of control plants (38.9 mm average as opposed to 40.46 mm for control). All other seedlings centrifuged continuously during the experimental period show a decreased

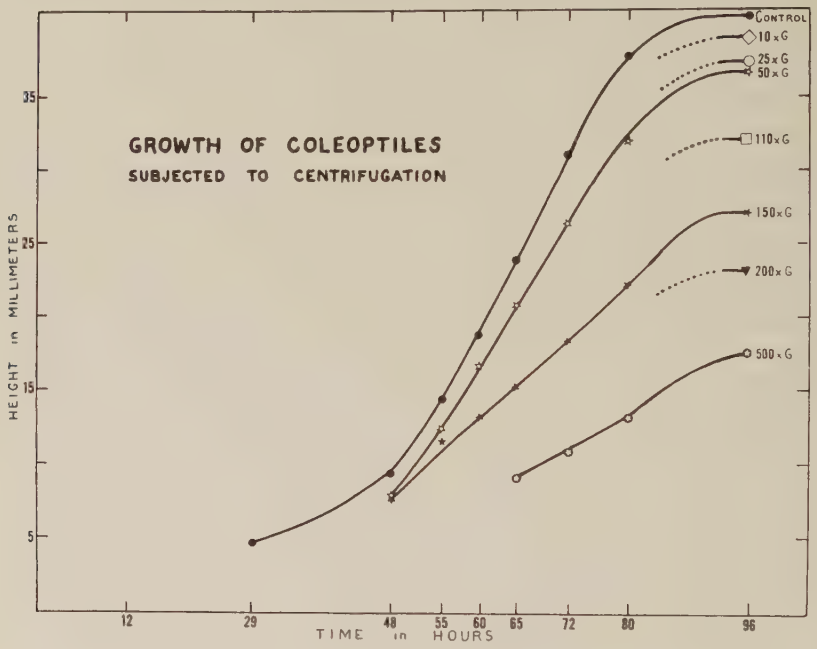


Fig. 2 Graph of coleoptile growth.

velocity of growth and a decrease in the total height attained. The decreased final height will not be appreciably changed by allowing the plants to grow for longer periods, for plants centrifuged at $500 \times G$ were allowed to grow for as much as 50 hours longer without showing further approach to the height of controls.

Diameters. Both the larger and smaller diameters of the oval coleoptile were measured with an ocular micrometer as

soon as the plant was removed from the sand. Both dimensions increase with age; the increase is rapid between 5 and 10 mm but slows as the coleoptile gets older. The enlargement is not proportional to the initial size of the diameter, but is an equal increment in both directions. The result is that the coleoptile becomes more nearly round with age.

Centrifugation increases diameter size and its rate of change. The initial size is greater, as is the final size attained, and the rate of increase is speeded up. The *proportional* increase, however, stays constant, so that the larger diameter is always about 0.43 mm greater than the smaller diameter.

TABLE 2

Changes in diameters and cross sectional area

TYPE PLANT (Number)	CONTROL (443)	10 × G (123)	25 × G (169)	50 × G (123)	150 × G (101)	500 × G (106)
Small diameter (mm)	1.140	1.208	1.215	1.268	1.374	1.462
Large diameter (mm)	1.578	1.654	1.630	1.712	1.778	1.899
Difference [1]	0.438	0.446	0.415	0.444	0.404	0.437
Ratio of diameters	0.722	0.730	0.745	0.741	0.773	0.770
Cross sectional area in mm^2	1.412	1.570	1.555	1.703	1.947	2.180
% of control area	100.0%	111.2%	110.1%	120.6%	137.9%	154.4%

[1] Average difference in diameters — 0.431 mm.

This means that a mature coleoptile grown at 500 × G has a ratio of diameters of 1:1.299 while in a mature control coleoptile the diameters are as 1:1.385. (The latter began life with a ratio of 1:1.154.) Thus the shape of the centrifuged coleoptile is shorter, thicker and more nearly round in cross section.

Table 2 shows actual changes in diameters in several types of plants, and figure 4 shows graphically the percentage relationship between experimental and control plants.

Area of cross section. These changes in diameter, of course, increase the cross-sectional area of the coleoptile and its resistance to bending forces (see table 2). Area of cross

section determined by diameters alone is slightly misleading, as the coleoptile is a hollow column. Least moments of inertia (I) were calculated, however, for hollow coleoptiles by the formula:

$$I = \frac{Pi}{4} \; a^2 \; (a - 3b)t$$

where I is the least moment of inertia (strength along the shorter diameter) and a is the small radius, b is the large radius and t is the least thickness of the coleoptile wall.

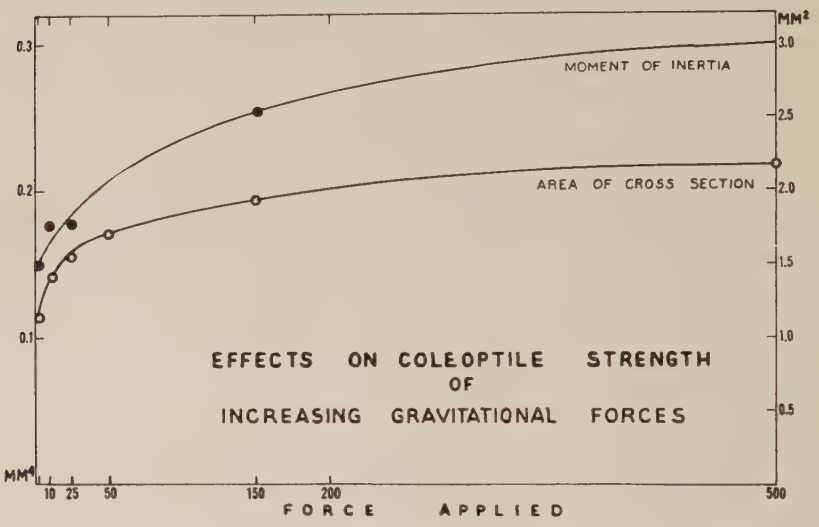


Fig. 3 Changes in area of cross section of coleoptile and in moment of inertia with increasing gravitational forces.

Between the control plants and those centrifuged, a consistent increase occurs up to 54.4% in cross sectional area and up to 100% in the moment of inertia at 500 × G. (See fig. 3.)

To determine if this size increase actually results in increased resistance to bending, segments of coleoptiles were tested in a tensiometer and the weight required for a deflection of 1 mm was determined. Bending moment was calculated as follows:

$$M_t = Fl - \frac{wl}{2}$$

where M is the total moment of bending, F is the force

required for deflection, l is the length of segment tested and w is weight of segment tested. These experiments showed that the enlarged diameter did result in greater resistance to bending in both directions. The expected increase in resistance between the control and 150 × G plants was 16.6% and that actually determined by tensiometer test was 15.4%. (See table 3 for other values.)

TABLE 3

Least moments of inertia and bending related to cross sectional areas in centrifuged and control plants

TYPE PLANT	CONTROL	10 × G	25 × G	50 × G	150 × G	500 × G
Area of cross section (mm^2)	1.412	1.570	1.555	1.703	1.947	2.180
Least moment of inertia (mm^4)	0.1500	0.1768	0.1770	0.1960	0.2486	0.3004
Least moment of bending [1] (gm cm)	0.2208			0.2994	0.3413	
Greatest moment of bending [2] (gm cm)	0.2727			0.3882	0.4590	

[1] In the direction of the small diameter.
[2] In the direction of the larger diameter.

Volume. In spite of decrease in the height attained by seedlings subjected to increased gravitational forces, the increase in cross sectional area allows the total volume of the epicotyl to remain roughly constant (around 60 mm^3) until the force applied exceeds 100 × G. Above this force, the increase in diameters no longer compensates for the decreased height. This is equally true if the coleoptile only is considered. Table 4 shows these changes for shoot volume computed as follows:

$$V_{shoot} = \left(\frac{D_1}{2}\frac{D_2}{2}\right) \text{Pi} \times \text{Height}$$

and

$$V_{coleoptile} = \left(\frac{D_1}{2}\frac{D_2}{2} - \frac{d_1}{2}\frac{d_2}{2}\right) \text{Pi} \times \text{Height}$$

where D is diameter of coleoptile and d is diameter of central lumen.

Weight. Weight of mature plants centrifuged at various forces is shown in table 5. In the growing plant, the shoot weight increases linearly with height, although plants less than 10 mm tall are slightly lighter in proportion to their size due to the absence of a leaf in the hollow center of the coleoptile.

TABLE 4

Volume of coleoptile and shoot

TYPE PLANT	CONTROL	10 × G	25 × G	50 × G	150 × G	500 × G
Vol. of coleoptile (mm^3)	41.23	42.50	40.54	42.13	34.49	27.13
Vol. of total shoot (mm^3)	57.13	61.10	57.94	62.38	52.63	38.30

The weight per millimeter of height does not rise with centrifugation, despite the increased diameters, until the force exceeds 150 × G. Indeed, at 25 × G and 50 × G the wet weight actually decreases, but this is not statistically significant. Dry weight rises, as does wet weight, but slightly faster, so that the percent of dry weight to wet weight

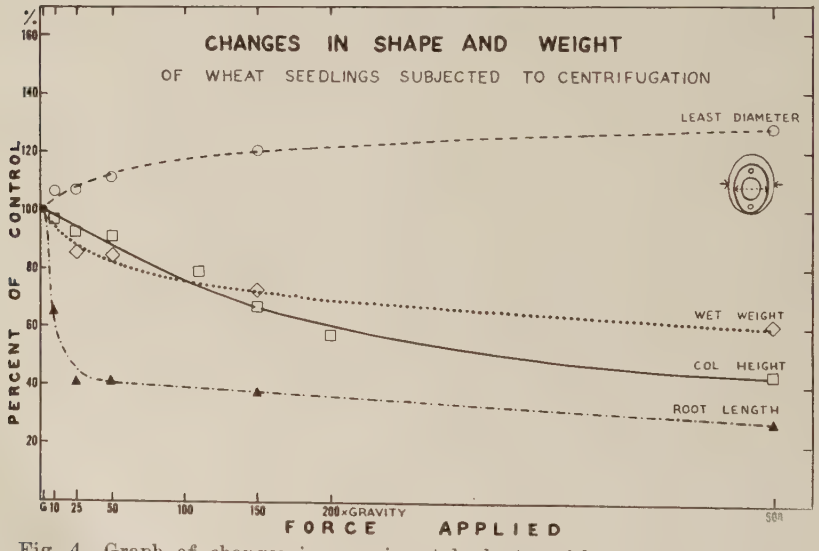


Fig. 4 Graph of changes in experimental plants, with control considered as 100%. Diagram shows proportional change of diameters at 500 × G over control.

increases in plants centrifuged at 500 × G. In spite of this increase of dry weight, there is no evidence of failure of water transport.

TABLE 5

Weight of entire shoot

TYPE PLANT (Number)	CONTROL (1 × G) (118)	25 × G (16)	50 × G (42)	150 × G (62)	500 × G (24)
Wet weight in mg	47.26	40.13	39.89	34.00	28.71
% of control		84.91%	84.41%	71.94%	60.75%
Wet weight/mm height	1.167	1.077	1.089	1.258	1.634
	± 0.232	± 0.125	± 0.155	± 0.172	± 0.173
% of control		92.2%	93.4%	107.8%	140.0%
Wet wt./mm³	0.8275	0.6940	0.6410	0.6460	0.7490
% of control		83.9%	77.5%	78.2%	90.5%
Dry wt./mm height	0.1090			0.1349	0.1878
	± 0.0205				± 0.0179
% of control				123.7%	172.2%
Dry wt. % of wet wt.	9.35%			10.72%	11.49%

Roots. When taken from the sand the roots of control seedlings were observed to be whitish and wavy, usually three in number. Under centrifugation the roots began to be tannish in color, stiffer, and more knotted or "kinky" in appearance. Gross differences became more evident as supragravitational forces increase; photographs (fig. 8) show the characteristic form with shorter roots on consistently shorter plants, but indicate only slightly the real difference in texture of roots of plants centrifuged at the higher speeds.

Roots were measured on plants of various heights in order to secure a picture of root growth in relation to height (and therefore time). Total root length was estimated by measuring the central root with a millimeter scale and multiplying this figure by the number of roots present (see Keeble et al., '31); admittedly an approximation, this method consistently applied gives comparable data for all plants.

The total root length bears a constant relation to shoot height during the experimental period in accordance with the relative growth equation of Huxley:

$$y = bx^k$$

where, in this case, y is the coleoptile height, x is the total length of roots, and k is the relative rate of growth. The growth of the coleoptile in the control is 0.412 mm for each 1.0 mm of root growth in the same length of time; hence,

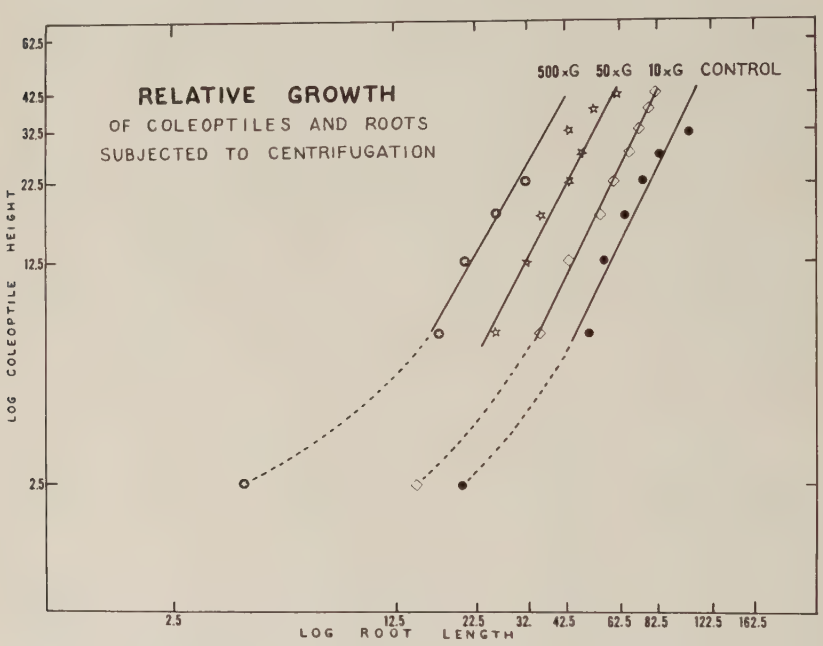


Fig. 5 Relative growth of coleoptiles and roots, plotted logarithmically.

the roots of the control plants grow about 2.4 times as fast as do the coleoptiles This value remains quite constant regardless of the centrifugal force applied; the altered factor is the value of b (see fig. 5).

In other words, the race between roots and coleoptiles is run more slowly as the force increases, but the initial handicap of the coleoptile is reduced and the two start more nearly even. Regardless of the centrifugal speed or the handicap, the roots will grow over twice as fast.

Average total root length at different heights is presented in table 6. Differences over 10 mm between any given lengths may be considered significant.

Microscopic observations. Longitudinal serial sections of representative plants of each type were prepared and studied.

TABLE 6

Root length in millimeters related to height of coleoptile

TYPE PLANT	CONTROL	10 × G	25 × G	50 × G	150 × G	500 × G
Col. height mm						
0–4	20.2 (9)[1]	14.6 (5)	7.2 (6)	23.0 (9)	6.7 (7)	4.3 (12)
5–9	50.5 (51)	35.3 (48)	24.5 (12)	25.8 (56)	12.4 (10)	17.2 (9)
10–14	56.6 (22)	43.9 (15)	33.5 (32)	32.3 (7)	25.6 (5)	20.8 (21)
15–19	65.7 (36)	54.6 (5)	38.9 (20)	36.0 (9)	31.0 (6)	25.8 (48)
20–24	76.8 (27)	60.2 (25)	40.7 (20)	44.0 (44)	44.6 (10)	31.9 (15)
25–29	83.8 (17)	67.1 (28)	44.0 (22)	48.0 (5)	45.4 (16)	
30–34	102.6 (51)	72.0 (7)	44.4 (22)	43.8 (10)	44.1 (7)	
35–39	118.2 (65)	79.0 (23)	50.0 (9)	52.1 (20)		
40–44	130.6 (41)	81.2 (20)	57.3 (12)	62.1 (24)		
45–49	167.7 (17)	83.8 (13)	61.4 (12)	60.0 (2)		

[1] Number of plants in parentheses.

No outstanding histological differences were observed between various experimental plants and the controls.

In all plants, the meristematic region at the first internode shows cells which have less cytoplasm in relation to nuclei of the same size in other cells. Except in this region, there

is a definite increase in length of cells as coleoptiles grow taller. Most of the cells in the tissues of coleoptiles over 5 mm high are in the mitotic resting stage, with an average complement of two nucleoli per nucleus. These did not show the tendency to centrifugal orientation in the nucleus which Kostoff ('37) observed in plant cells at forces above 1500 × G.

Since length of cell is related to the shoot height, parenchyma cells were measured at the base of coleoptiles of known

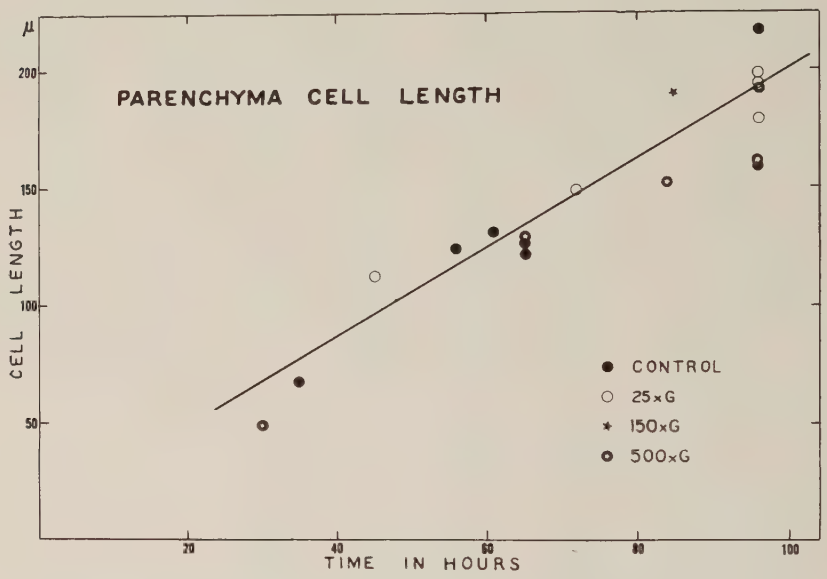


Fig. 6 Relation of parenchyma cell length to age of coleoptile. Each point represents an average of the cell lengths in a single plant.

height. In the control plants, these cells become longer but not appreciably wider as the coleoptile grows taller. In plants grown under centrifugal forces, both cell length and cell diameter increase markedly in comparison with cells of control coleoptiles of the same height (see fig. 6). With respect to time, however, there is little difference between cell length attained; at the end of the growing period cells in a 500 × G plant are about the same length as cells in the

control plant. There are merely fewer cells and the coleoptile is shorter at the end of the 96 hour period.

The increase in diameter of the parenchyma cells reminds one that the whole coleoptile diameter increased with the force applied. (Table 2 and fig. 4.) Comparison of figures shows that increase in cell diameter is more than sufficient to account for coleoptile enlargement; there are usually fewer cells in the cross section as well as in the length of centrifuged coleoptiles.

No visible changes accounted for the increase in relative dry weight. The cell walls were not seen to be any thicker in the centrifuged plants, and, as cell length is unchanged

TABLE 7

Mortality and germination rates of control and experimental plants

TYPE PLANT	GERMINATION	MORTALITY
	%	%
Control (1 × G)	74.7	25.3
25 × G	52.8	47.2
50 × G	43.7	56.3
150 × G	43.3	56.7
500 × G	23.9	76.1

there seem to be no additional cell walls present to account for additional material.

Mortality. That the centrifugal forces applied to the plants provided conditions of stress was evidenced by the germination figures. While germination varied from one year's crop of seeds to the next, a consistent decline in germination rate was found with increasing speed of centrifugation (see table 7).

DISCUSSION

Changes in form. The changes taking place in the shape of the wheat seedling as the result of increased gravitational forces are much more than an overall retardation of development. Thus the centrifuged coleoptile is progressively shorter, stouter, and more round, has fewer cells and is served by

shorter roots; yet these changes are by no means proportional to each other.

Furthermore, most of the changes are not proportional to the force employed. Thus the inhibition of total height attained at 50 × G amounts to 9.5% of control height, yet 10 times this force, 500 ×G, produces 56.6% inhibition, which is by no means 10 times the effect produced at 50 × G. (See table 8.) The diameters respond similarly, although their response is an increase in size, rather than a decrease. Area of cross section increases 20% over the control when the coleoptile is subjected to 50 × G, but 500 × G produces only

TABLE 8

Inhibition of height of experimental plants subjected to supragravitational forces

FORCE	FINAL HEIGHT	% OF CONTROL HEIGHT	% INHIBITION
	average		
Control (1 × G)	40.46	100.0	none
10 × G	38.92	96.3	3.7
25 × G	37.26	92.1	7.4
50 × G	36.63	90.5	9.5
110 × G	31.98	79.1	20.9
150 × G	27.03	66.8	33.2
200 × G	23.10	57.1	42.9
500 × G	17.57	43.4	56.6

54% total increase. Only the root length responds proportionally to the forces applied, decreasing with the logarithm of the gravitational field.

Root length shows a slight tendency toward inhibition after attaining 40 to 45 mm of length. This is not clearly marked and would have escaped attention had it not been for the work of Quastler, Schertiger and Stewart ('52). These investigators found that bean roots after x-radiation show arrest of growth "after certain well-defined root lengths have been reached." Unlike the beans, our wheat seedlings showed a response graded to the force applied, but at 25 × G, 50 × G and 150 × G they showed a slowing of growth of roots when the coleoptile was between 20 and 35 mm tall. (See table

6.) As no mitotic counts on roots were made, it is not possible to say whether this retardation is the same effect as that observed by Quastler et al.

Lest it be felt that the roots in growing downward should be aided by gravity in this growth, it should be remembered that the roots as well as the coleoptile must do work against, not with, the effects of the earth's attraction. For each cubic millimeter of space they occupy, an equal amount of water and substrate must have been displaced upward toward the free surface. Under centrifugation this is accomplished with increasing difficulty as the effective weight of the overlying substrate increases, as each particle to be displaced weighs more in proportion to the centrifugal speed.

In centrifuged plants, structural changes might be expected as the force of effective weight approaches the bending limit of the tissues of the coleoptile. A change in form toward an increase in cross sectional area would give additional strength for a given height and diameter ratio, or a less elliptical shape of the same area would achieve a similar result. An increase in diameters, which would serve to strengthen the column of the coleoptile, does occur, apparently as an adaptive response. The relatively small increase resulting from greatly increased forces is probably an inherent limit of monocotyledonous growth. Certainly, the experimental coleoptiles are closer to mechanical failure than are the controls, for the increase in diameters resulting from centrifugation does not compensate for the increased effective weight.

Although some changes take place at forces as low as $10 \times G$, these changes are slight. Still lower forces, not here reported in detail, show no visible effects. There is no evidence that variations in the gravitational constant of the earth at different points on its surface would have an appreciable effect upon growing plants; while such differences do exist, they amount to only about 0.5% of the value of G.

Work. At the beginning of this research, a fundamental question to be answered was: What response would be made

by the plant to the extra work entailed in vertical growth aganist supragravitational forces? These possibilities exist:

1. The plant would do the same amount of work it does normally, and its final height would be much less;
2. It would do proportionately more work to attain the normal height; or,
3. It would strike a compromise between (1) and (2).

Each particle making up the coleoptile is the result of work done in lifting it from the level of the planted seed to the position it occupies. Work equals force times the distance moved. By assuming the coleoptile to be of constant diameter throughout its height (which it very nearly is), the force overcome is that of gravity times the weight of the material raised vertically. The average distance through which the particles are moved is the height of the center of gravity of the coleoptile, or one-half of its actual height. Thus we have:

$$\text{Work} = \text{Weight} \times \text{G} \times \tfrac{1}{2} \text{ Height}$$

and applying this formula to the control plants, we have:

$$\text{Work} = 0.04726 \text{ gm} \times 1\text{G} \times \frac{4.046 \text{ cm}}{2} = 0.095607 \text{ gm cm}$$

The work done by the plants centrifuged at various forces is shown in figure 7 and table 9. It will be seen that the work output increases only slightly less than proportionally up to $50 \times$G. In other words, at $50 \times$ G the plant does almost 50 times as much work as does the control. Beyond $100 \times$ G the compensation falls off markedly. At $500 \times$ G the work accomplished is only about 132 times that done by the control plant. It is obvious then, that the second possibility considered above is the case for the lower centrifugal forces applied, and that the third proposition holds true for forces above $100 \times$ G.

While the work remains proportional to the lower forces applied, it must be noted that the increased diameters of the centrifuged plants make up, in part, for the lower height attained. The efficiency picture has changed toward moving a greater weight a shorter distance.

The curve of work shown in figure 7 does not by any means represent the total work done by the plant. The raising of

water to be transpired requires increasing work with increasing gravitational force; as the quantity transpired was not determined, this work is not measurable. The roots, as mentioned, also do work as they elongate in the substrate; it was

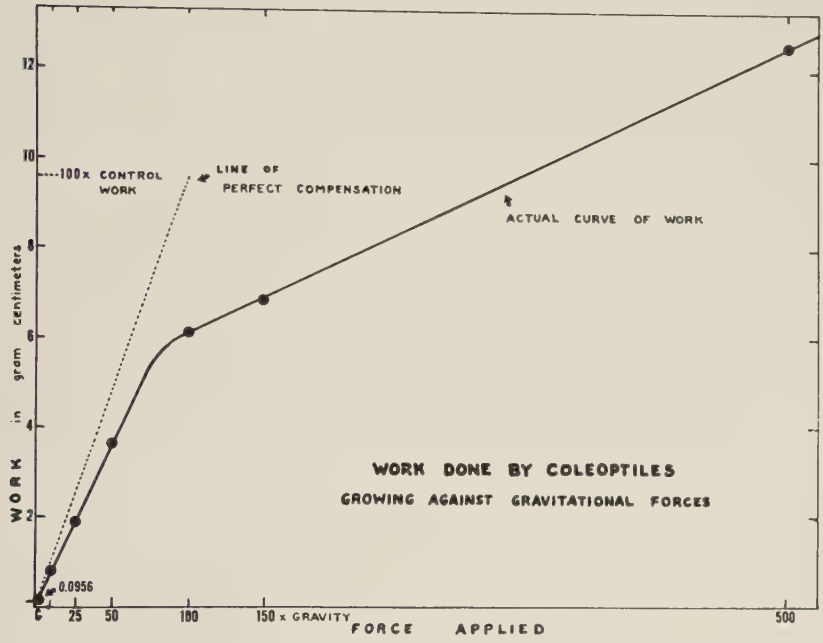


Fig. 7 Work done by coleoptiles growing against different gravitational forces.

TABLE 9

Work done by growing shoot in response to increased gravitational force

FORCE	MATURE HEIGHT	MATURE WEIGHT	EFFECTIVE WEIGHT	WORK ACCOMPLISHED		% PERFECT COMPENSATION
				gm cm	× control	
	mm	*mg*	*mg*			
Control	40.46	47.26	47.26	0.0956	1 × Control	
10 × G	38.92	42.50	425.00	0.8271	8.65 × C	86.5
25 × G	37.26	40.13	1003.25	1.8691	19.55 × C	78.2
50 × G	36.63	39.89	1994.50	3.6539	38.20 × C	76.4
100 × G	32.13	38.10	3810.00	6.1227	64.15 × C	64.2
150 × G	27.03	34.00	5100.00	6.8952	72.10 × C	48.1
500 × G	17.57	28.71	14355.00	12.6180	131.99 × C	26.4

not practicable to measure this work. Other processes requiring energy go on in a living plant, many of which are probably not primarily affected by gravity; these were not considered in our work curve. It should be mentioned that a wheat seed contains slightly over 0.1 kilocalorie or the equivalent of 5,500,000 gram-centimeters of work. The percent of this total expended for coleoptile growth, even at 500 ×G, is thus very small.

Cell number. It remains to be considered how the coleoptile comes to be shorter in the experimental plants.

From measurements of parenchymal cell length, it seems that in the same length of time equal elongation takes place, regardless of the gravitational force. Therefore a shorter mature coleoptile must mean fewer cells. At 96 hours, a 40 mm control coleoptile has parenchyma cells of about 180 μ in length, or about 222 cells in a column 40 mm long. A 500 × G coleoptile of the same age has cells about 193 μ long, with about 104 cells in a similar column only 22 mm tall. Being half as tall, it takes half as many cells to build it. (On the other hand, the parenchyma cells of a 20 mm control plant, usually 61 hours old, are still elongating and the largest are only 133 μ long.)

The normal elongation of the parenchyma cells in the centrifuged plants indicates that even though auxins may move faster through the tissues due to increased forces of gravity, the elongation of cells proceeds at the same rate and to the same final length as in control plants. Pfaeltzer ('34) found no indication of the effect of gravity on longitudinal transport of auxin in *Avena,* and Went ('26), Dolk ('30) and Pfaeltzer found that auxin production was unaffected by the direction of action of gravity.

Decreased height is not the only factor: the centrifuged coleoptile has increased in diameter, as have its cells. The total cross sectional area (less the hollow center) of the control coleoptile is 1.018 mm^2 and that of the 500 ×G coleoptile is 1.1276 mm^2, while the diameters of the parenchymal cells are 25.07 μ and 27.79 μ respectively. The cellular cross section

areas are 469 μ^2, and 577 μ^2 when calculated as hexagons. Thus the control coleoptile has an area of cross section equal to 2170 cell areas, while the 500 $\times$ G coleoptile has an area equal to 2212 cells and this difference is not significant. The increased diameter, therefore, will not account for the missing cells.

These results suggest that the inhibition of linear growth must concern cell division and not cell elongation. Using much higher forces, Hilbe ('42) found that division of root tip cells was inhibited at metaphase, but this resulted in tetraploid cells of greatly increased size, or in some binucleate and some non-nucleate cells. Such cells were not observed in our material. The alternative is that many cells, although formed, failed to elongate, while other elongated normally. No evidence for this hypothesis exists in the meristematic area at the base of the coleoptile. The "missing" cells must never have been produced.

SUMMARY AND CONCLUSIONS

1. Wheat coleoptiles grown in the dark were centrifuged at various forces between 10 $\times$ G and 500 $\times$ G, with resulting structural changes.

2. Total height attained, decreased with increasing gravitational force, but the time required for total height to be reached was unchanged.

3. Diameters increased with the force employed, with resulting increased resistance to bending.

4. Root length decreased logarithmically with increased force.

5. The work done by the plant in growing its coleoptile increases nearly proportionally to the increased force applied, up to 100 $\times$ G beyond which the work, while still increasing, is no longer compensatory.

6. The work done in raising the shoot to mature coleoptile height, even under forces of 500 $\times$ G, is negligible in comparison with the total seed energy reserve.

7. Parenchyma cell length was not affected, but cell width increased. No cytological changes were observed.

8. As coleoptile size decreased without a corresponding decrease in cell size, it is assumed that cell division is inhibited by supranormal gravitational forces, while cell elongation is not affected.

9. Variations in the gravitational attraction at the surface of the earth are far too slight to produce any effects upon the seedling.

LITERATURE CITED

Avery, G. S., Jr. 1930 Comparative anatomy and morphology of embryos and seedlings of maize, oats and wheat. Bot. Gaz., *89:* 1–39.

Beams, H. W., and R. L. King 1944 Effects of ultracentrifuging on polarity in pollen grains of *Vinca rosa.* J. Cell. and Comp. Physiol., *24:* 109–114.

Boyd, L., and G. S. Avery, Jr. 1936 Grass seedling anatomy: The first internode of *Avena* and *Triticum.* Bot. Gaz., *97:* 765–779.

Camara, A. 1942 Variacoes cromosomicas estruturais induzidas pela centrifugacao. Agronomia Lusitana, *4* (3): 199–211.

Dolk, H. E. 1930 Geotropie en groeistof. Diss. Utrecht. *Cited in* Went and Thimann.

Conklin, E. G. 1917 Effects of centrifugal force on the structure and development of the eggs of *Crepidula.* J. Exp. Zool., *22:* 311–419.

———— 1931 The development of centrifuged eggs of ascidians. J. Exp. Zool., *60:* 1–120.

Gray, S. W., and R. G. Webb 1950 Effect of supranormal gravitational force on swimming habits of tadpoles. Anat. Rec., *108:* 131.

Harvey, E. N. 1931 Observations on living cells made with the microscope-centrifuge. J. Exp. Biol., *8:* 267.

———— 1934 The air turbine for high speed centrifuging of biological material, together with some observations on centrifuged eggs. Biol. Bull., *66:* 48–54.

Heilbrunn, L. V. 1943 An Outline of General Physiology. W. B. Saunders Co., Phila.

Hertwig, O. 1899 Ueber einige durch Centrifugelkraft in der Entwicklung des Frocheies hervorgerufene Varanderungen. Arch. f. Mikrosk. Anat., *53:* 415–444.

Hilbe, J. J. 1942 The effect of ultracentrifuging germinating seeds of onion and rye. Proc. Iowa Acad. Sci., *48:* 457–466.

Keeble, F. M., M. G. Nelson and R. Snow 1931 The integration of plant behavior. III. The effect of gravity on the growth of roots. Proc. Roy. Soc. Lond. B, *108:* 360–365.

Knight, T. A. 1806 On the direction of the radicle and germen during the vegetation of seeds. Phil. Trans. Roy. Soc., *96:* 99–108.

KOSTOFF, D. 1927 The effect of centrifuging upon the germinated seeds from various plants. Cytologica, *8:* 420–442.

MCCALL, M. A. 1934 Developmental anatomy and homologies in wheat. J. Agric. Res., *48:* 283–321.

PFAELTZER, J. W. 1934 Lengtekracht, groeistof en groei bij het coleoptiel van *Avena sativa.* Diss. Utrecht. *Cited in* Went and Thimann.

QUASTLER, H., A. M. SCHERTIGER AND W. N. STEWART 1952 Inhibition of plant growth by irradiation. IV. Growth arrest vs. effects on mitotic activity. J. Cell. and Comp. Physiol., *39:* 357–370.

RANDOLPH, L. F. 1935 A new fixing fluid and a revised schedule for the paraffin method in plant cytology. Stain Tech., *10:* 95–96.

SAEZ, F. A. 1941 Alteraciones experimentles induceidas por la accion de la gravedad on las celulas somaticas de *Lathyrus odoratus.* An. Soc. Cient. Argentina, *132* (4): 139–150.

SCHECTER, V. 1935 The effect of centrifuging on the polarity of an alga, *Griffithsia bornetiana,* Biol. Bull., *68:* 172–179.

THOMPSON, D'ARCY W. 1942 On Growth and Form. MacMillan Co., New York. p. 78.

TODD, R. E. 1940 The cellular components of centrifuged frog eggs. J. Exp. Zool., *83:* 359–378.

WENT, F. W. 1926 On growth accelerating substances in the coleoptile of *Avena sativa.* Proc. Ken. Akad. Wetensch., *30:* 10–19.

———— 1954 Dynamics of Growth Processes. Ed. by Edgar J. Boell, Princeton University Press. Chap. VII. Physical factors affecting growth in plants. pp. 130–147.

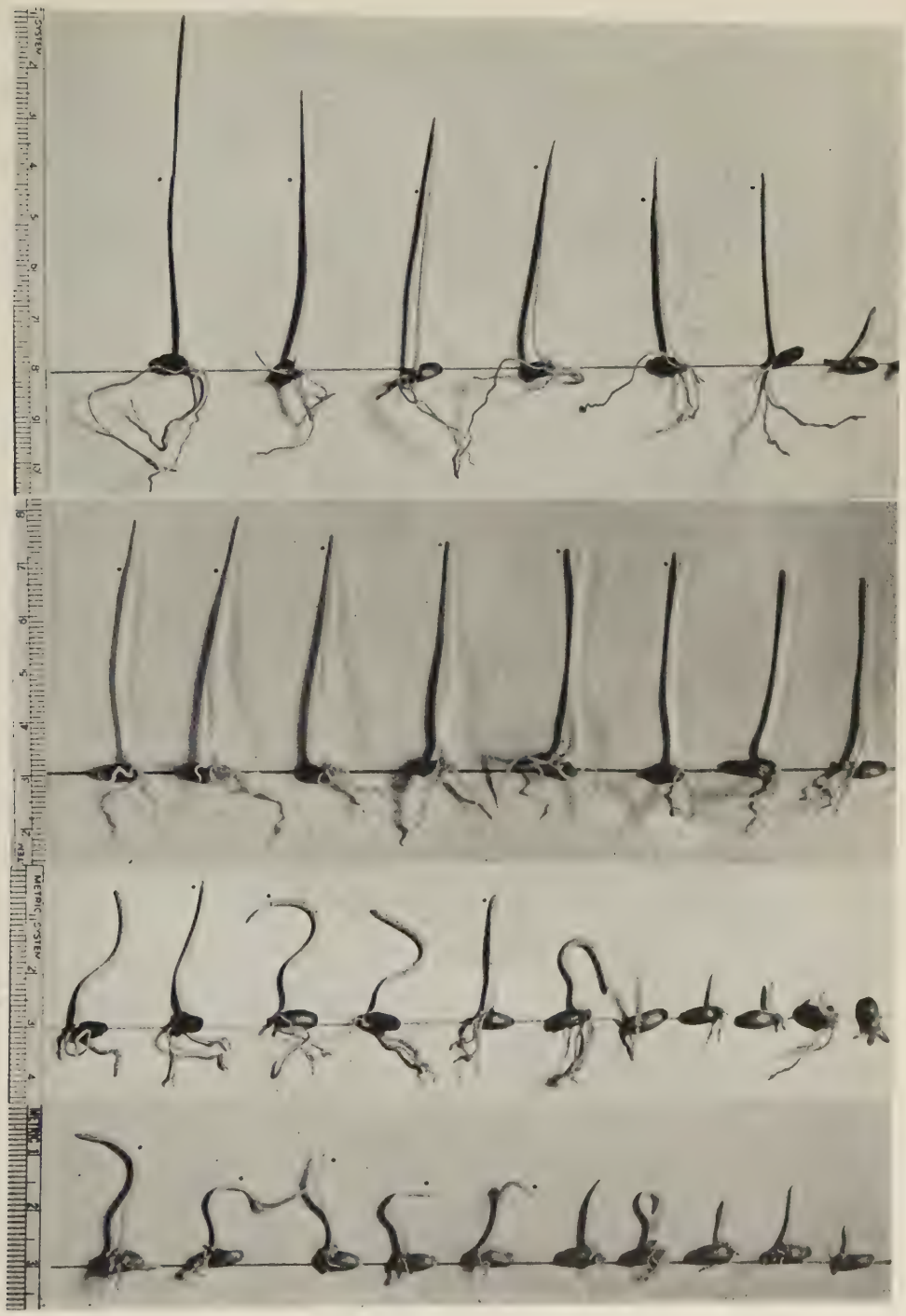
WENT, F. W., AND K. V. THIMANN 1937 Phytohormones. The Macmillan Co. New York.

PLATE 1

EXPLANATION OF FIGURES

8 Typical wheat seedlings, uprooted at 96 hours. The dots indicate height of tip of coleoptiles through which first leaf has emerged.

Top row: Control (1 × G) seedlings.
Second row: plants grown at 50 × G.
Third row: plants grown at 150 × G.
Bottom row: plants grown at 500 × G.



MECHANISMS INFLUENCING THE PERMEABILITY OF THE BLOOD-BRAIN BARRIER [1]

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A review of the literature dealing with the blood-brain barrier indicates that numerous attempts have been made in the past to influence the passage of various substances into the brain. Much of this work has been of a qualitative nature as, for example, most of the work with the supravital dyes. Another large segment of studies has been concerned with isolated attempts to modify the penetration of a single substance into the central nervous system. Since it has been shown that a certain drug or treatment may have variable and even opposite permeability effects, depending upon the substance that is used as a tracer agent (Stern and Lokchina, '27; Stern, Kassil, and Lokchina, '27; Hurst and Davies, '50), it would appear essential that cerebrovascular permeability be studied using a variety of test molecules.

The present report deals with a series of studies in which we have attempted to modify the permeability of the blood-brain barrier by physiological and pharmacological methods as judged by two members of the sulfonamide group, namely, sulfapyridine and sulfanilamide, used as tracer agents. These agents were used inasmuch as a previous study had shown the rates of permeation of these compounds into the brain to be widely divergent (Goldsworthy, Aird, and Becker, '54).

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METHODS

Control animals, consisting of male rats of the Long-Evans strain and weighing approximately 300 gm, were injected intraperitoneally with either 6.0 ml of an isotonic 0.8% suspension of sulfapyridine per rat or with 2.0 ml of an isotonic 0.8% solution of sulfanilamide per 100 gm weight of rat. The blood, plasma, and brain concentrations of sulfonamide were determined after a one-hour period and the data so obtained was used to compute ratios of brain-to-blood or brain-to-plasma concentrations. The details of the procedure were essentially as presented in a previous paper (Goldsworthy, Aird, and Becker, '54). It should be noted that the method involved rate measurements rather than equilibrium or "steady state" determinations. In the present studies, all blood and plasma samples were deproteinized at a dilution by volume of 1:50; brain samples in the sulfapyridine experiments were deproteinized at a dilution by weight of 1:25; and in the case of the sulfanilamide determinations, at a dilution by weight of 1:100. At the latter dilution, with the relatively high concentrations of brain sulfanilamide obtained, a reproducible recovery of 93% was achieved. A correctional factor consequently was applied.

As indicated in our previous studies (Goldsworthy, Aird, and Becker, '54), a wide variation in the sulfonamide dosage produced no change in the brain-to-blood or brain-to-plasma ratios. Nevertheless, sulfanilamide given on a weight basis afforded an indication of the reproducibility of concentrations that was possible from animal to animal. Thus, the mean concentration of sulfanilamide in the brains of 16 control animals was 8.8 mg% with a standard deviation of only $\pm$ 0.25 mg%. Control experiments were not done sequentially, but were interspersed throughout the whole series of experiments. Another index of the reliability of the method was indicated by the essentially linear relationship of the brain, blood, and plasma concentrations of sulfanilamide to the dosage of sulfanilamide when it was injected over a dosage range of from two to 20 mg per 100 gm of body weight. It is of interest

in this connection that Hurst and Davies ('50) found considerable variation from animal to animal (mice) in the brain concentrations of sulfanilamide and sulfanilic acid, when these compounds in 0.1 ml of water were injected intravenously, with sacrifice after 20 minutes. It would appear that their more variable results might be explained by the short time intervals they used and the fact that they did not relate the brain concentrations to a blood or plasma reference concentration.

Aside from the administration of the drug or treatment used in the attempt to modify cerebrovascular permeability, all test animals were handled precisely in the same fashion as the control animals. The methods used are shown in table 1 and were selected in each case for reasons indicated below under "Results and Discussion." The measures used were initiated at a time calculated to give a maximal effect during the period of high blood sulfonamide concentration. In most cases a range of different drug concentrations was studied at different times of administration in an attempt to establish as firmly as possible whether or not the drug or treatment used had any effect upon the rate of permeation of these sulfonamides into the brain.

RESULTS AND DISCUSSION

The results summarized in table 1 represent an initial survey of agents or measures which were selected because of their possible effect in modifying cerebrovascular permeability. Although many of the effects noted cannot be ascribed to permeability changes alone, the survey is of value in drawing attention to agents or treatments worthy of further study. For the most part, drastic attempts to modify the course of sulfonamide distribution were avoided. The results, in general, indicate that cerebrovascular permeability can be altered only with difficulty when moderate or "physiological" methods are used.

In presenting this data it is fully appreciated that any final interpretation of the results is limited by the relatively

TABLE 1

Measures used in an attempt to modify cerebrovascular permeability

TREATMENT	SULFAPYRIDINE				SULFANILAMIDE			
The intervals of time are indicated before (+) and after (−) sulfonamide injection. IP = intraperitoneal. Subc. = subcutaneous.	No. of animals	Brain / Plasma Ratio [1] ± Stand. dev.	% Diff. from control	p value [3]	No. of animals	Brain / Plasma Ratio [1] ± Stand. dev.	% Diff. from control	p value [3]
Control group	14	0.26 ± .03	..	..	14	0.69 ± .02	..	..
Acidosis — NH_4Cl 275–300 mg/kg IP at + 10 min.	16	0.26 ± .03	..	..	6	0.60 ± .03	— 13%	< .001
Alkalosis — $NaHCO_3$ 1 mg/kg IP at + 10 min.	4	0.26 ± .02	..	..	7	0.75 ± .03	+ 9%	< .001
Dehydration — 3 days no water. Av. wt. loss, 14%	3	0.27	..	..	6	0.71 ± .02	..	..
Dehydration — 2 days no water. Av. wt. loss, 15%. 28% glucose 10 ml/kg IP at + 5 min.	4	0.22 ± .02	— 15%	.02				
Dehydration — 3 days no water. Av. wt. loss, 16%. 30% glucose 20 ml/kg IP with sulfonamide	9	0.30 [2] ± .02	+ 15%	< .001	6	0.53 [2] ± .03	— 15%	< .001
Hydration — 0.5% NaCl 60 ml/kg IP at + 15 min.					8	0.66 ± .05	..	..
Adrenalin — 1.0 mg/kg subc. at + 5 min.	6	0.23 ± .05	— 12%	< .10	6	0.53 ± .10	— 23%	< .001
$NaNO_2$ — 10 mg/kg subc. at + 10 min.	5	0.28 ± .04	..	..	6	0.75 ± .03	+ 9%	< .001
Metrazol — 20 to 35 mg/kg. IP at — 20 to — 45 min. (sulfapyridine) and 30 mg/kg IP					6	0.60 ± .03		

Aminophylline — 116 to 166 mg/kg IP at + 20 min.					7	0.61 ± .02	— 12%	< .001
Combination 3-day dehydration, atropine sulfate, 1.33 mg/kg IP at + 60 min.; aminophylline, 83 mg/kg IP at + 15 min.					7	0.60 ± .02	— 13%	< .001
Trypan red — 1 inj./day IP of 1% solution for 3 days for a total of 120 mg/kg. Sacrifice 3 days after final injection	7	0.28 ± .02	..	..	7	0.71 ± .03	..	..
Trypan red — 1 inj./day IP of 1% solution for 4 days, for a total of 200 mg/kg. Sacrifice 3 days after final injection	4	0.42 ± .02	+ 62%	< .001	4	0.78 ± .04	+ 13%	< .001
Sodium salicylate — 250 mg/kg subc. at + 10 min.					8	0.73 ± .05	+ 6%	< .05
Insulin-protamin-zinc (Lilly) 25 units/kg subc. at + 20 hrs. Ad. lib. feeding					7	0.72 ± .05	..	..
Acetylcholine chloride — 50 mg/kg subc. at — 30 min.	8	0.20 ± .01	— 23%	< .001	5	0.68 ± .05	..	..
Physostigmine sulfate — 0.8 mg/kg IP at — 20 min.	8	0.27 ± .03	..	..	7	0.77 ± .03	+ 12%	< .001

[1] Brain-to-blood ratios provided similar information as the brain-to-plasma ratios, and are not included for the sake of simplicity.

[2] The values for drastic dehydration are brain-to-blood values and should be compared to control values of 0.35 and 0.62 for sulfapyridine and sulfanilamide respectively.

[3] The statistical analysis was made as follows: $s^2 = \frac{\Sigma \text{ dev}^2 \text{ (exper.)} + \Sigma \text{ dev}^2 \text{ (control)}}{N \text{ (exp.)} + N \text{ (cont.)} - 2}$ $\quad t = \frac{\text{mean (exp.)} - \text{mean (cont.)}}{s \sqrt{\frac{N \text{ exp.} + N \text{ cont.}}{(N \text{ exp.})(N \text{ cont.})}}}$

unknown effects of the drugs or treatments upon (1) cerebral blood flow, and (2) the distribution of the tracers after parenteral injection as indicated by their plasma concentration against time curves. Certain assumptions, however, can be made regarding the latter factor. With sulfanilamide, the peak plasma concentration occurred about one-half hour after injection in control animals. In treated animals showing a decreased brain-to-plasma ratio, the decrease in the main could be ascribed to lower brain concentrations, although the plasma values at sacrifice also tended to be higher than control plasma values. This latter effect suggests a generalized decrease in the rate of sulfanilamide distribution. Thus, a slower absorption of the tracer into the blood stream would cause a shift of the peak plasma concentration to a later period nearer the time of sacrifice. Similarly, a slower distribution of the tracer into the tissues would act to sustain the plasma levels. In those experiments in which the brain-to-plasma ratios were higher than in the control animals, the increased ratios were found to be due to increased brain concentrations, except in the experiments in which sodium bicarbonate was used. In the bicarbonate experiments, plasma sulfanilamide concentrations were found which were slightly below control values.

The greatest effect on cerebrovascular permeability was found after intense trypan red staining, when sulfapyridine was used. A smaller increase in the sulfanilamide ratio was obtained under similar test conditions. However, when moderate staining levels of trypan red were employed, little or no effect was noted with either test sulfonamide. This result is in contrast to the findings of Aird and Strait ('44), who observed a significant decrease in the concentration of cocaine passing into the brain and cerebrospinal fluid as compared with control values, after similar, moderate trypan red staining. The blood concentrations of cocaine remained essentially the same in both control and treated animals. As a result of these studies, based upon the use of cocaine as a tracer

agent, it was concluded that trypan red exerted an effect on the permeability of the cerebral capillaries.

An interesting explanation for the different effects of moderate trypan red staining upon cocaine and the two sulfonamides may possibly be found in the differences in the lipid solubility and charge of these tracer agents. Pappenheimer, Renkin and Barrero ('51) adduced important evidence that lipid insoluble substances and water pass largely or solely through pores in the capillary wall which make up less than 0.2% of the histological surface of peripheral capillaries in the cat. (Cf. also Pappenheimer, '53). Renkin ('52), on the other hand, provided evidence supporting the view that lipid soluble molecules pass through the cellular membranes of the capillary endothelial cells. The danger of extending these results obtained on peripheral capillaries to the capillaries of the brain is obvious. Nevertheless, since cells in general are permeable to lipid soluble molecules, it appears likely that the lipid soluble cocaine passed through the capillary endothelial cells of the brain. Since trypan red stains the capillary endothelium (Aird and Strait, '44) and like brilliant vital red may be seen with supravital staining techniques to be deposited in the cytoplasm of the endothelial cells (but does not enter into the central nervous system proper), its effect upon cocaine presumably would result from some alteration in the environment of the endothelial cells, causing a decreased entry of cocaine into the brain.

Sulfapyridine, on the other hand, is only very slightly soluble in ether. Sulfanilamide, although soluble in ether, is insoluble in chloroform and petroleum ether. Again, Goldsworthy et al. ('54) showed a correlation between sulfonamide charge and the rate of permeation into rat brain, but no correlation between their lipid solubility and the rate of permeation of the sulfonamides into the brain. Thus, it seems probable that weakly acidic sulfonamides do not pass through the endothelial cells to any great extent, but rather through the pores in accordance with the observations of Pappenheimer et al. ('51). If this is correct, moderate trypan red

staining should have little effect on the passage of the slightly charged sulfonamides into the brain, as indeed was found to be the case. The increased passage of the sulfonamides with intense trypan red staining was interpreted as a toxic effect, inasmuch as this was suggested by the post-mortem examination of the tissues in the excessively stained animals.

Teorell ('49) has emphasized the importance of membrane potentials in studies utilizing charged particles, and noted that a potential difference of 58 millivolts will induce a tenfold separation of univalent charged particles, with a correspondingly greater effect as the charge per particle increases. Tschirgi and Taylor ('53, '54) observed a steady potential difference of 5–30 mv between venous blood and the cerebrospinal fluid (negative) of the cisterna magna in lightly anesthetized rats, cats, and dogs. They suggested that the potential was due to the activity of some pan-vascular membrane between the blood and cerebrospinal fluid, or between the blood and interstitial fluid compartment of the brain, and was not due to neuronal polarization. Although their observations give no verification of a similar potential between blood and interstitial fluid in the unanesthetized animal, the findings of Goldsworthy et al. ('54) could be explained in part on the basis of a similar membrane potential between the blood and extracellular fluid (negative) of the brain. The studies summarized by Friedemann ('42) also would be consistent with this view. At the pH of physiological solutions, the small positive charge carried by cocaine and the very slight negative charge of sulfanilamide would result in a negligible effect of membrane potentials upon their permeability. Sulfapyridine, on the other hand, is sufficiently acidic to be partially affected by such considerations.

In the case of rats injected with trypan red, another factor affecting the membrane potential must be considered. Trypan red, since it contains 5 sulfonic acid groups per molecule, is quite negative at the pH of biological fluids. This negativity, in combination with the impermeability of the cerebral capillaries to trypan red, favors the passage of diffusable negative

particles across the capillary wall in accordance with the principles of the Donnan equilibrium. Since the blood proteins are also negative, the trypan red Donnan effect would be additive to that of the blood proteins. Such an added effect, if it is of sufficient magnitude, should decrease the rate of permeation of positively charged particles, while increasing that of negatively charged ones. The acidity of the two sulfonamides used in this experiment, however, was inadequate to provide evidence upon this point.

Changes in pH and water balance were tested by use of the two sulfonamides because of their possible effect on permeability and their influence on convulsive reactivity. Although compounds disrupting pH regulation had no effect on the permeation of sulfapyridine, alkalosis caused an increased rate of passage of sulfanilamide into the brain, while acidosis caused a decrease. The results indicate that the effect of pH is other than an effect upon the charge of the test sulfonamide, since small physiological pH changes might be important to the charge of sulfapyridine (pKa of 8.43) but not to that of sulfanilamide (pKa of 10.43). Geiger and Magnes ('47) observed that only extreme changes of pH had any effect on the isolated cerebral blood flow of living cats during perfusion with ox blood, whereas, with the same preparation, Magnes and Geiger ('48) found that a pH change from 7.25 to 7.20 usually increased oxygen consumption 25–30%. These results suggest the possibility that metabolic work may be involved in the permeability of sulfanilamide. Drastic dehydration was effective in decreasing the passage of both tracers into the brain. The increased viscosity of the blood in the case of dehydration was probably a factor in the decreased rate of their distribution. Hydration (0.5% NaCl), however, had no significant effect on the sulfanilamide brain-to-blood ratio. Broman and Lindberg-Broman ('45) observed that changes of osmotic pressure (distilled water, hypertonic salt solutions) and of pH had no effect on the permeability of the cerebral vessels to trypan blue unless the changes were fairly great.

Agents known to affect the vascular system were used in order to determine what effect on permeability they might produce. Results obtained with the use of adrenalin were the most variable of all the measures tested (cf. table), especially when sulfanilamide was the tracer agent used. Yet, a significant decrease in the brain-to-plasma ratio was observed after adrenalin in the sulfanilamide experiments, while the decrease noted in the case of sulfapyridine was not significant. Sodium nitrite caused a small increase in the rate of penetration of sulfanilamide into the brain, but had no significant effect upon the permeation of sulfapyridine.

The effects of these two drugs upon blood flow obviously must be considered in interpreting the changes noted. The results obtained might be explained in terms of the vasoconstrictive effects of adrenalin and the vasodilator action of sodium nitrite, without implicating permeability effects, such as have been reported in the literature. Thus, adrenalin alone, or adrenalin in combination with pituitrin is generally thought to increase the cerebrovascular permeability of those substances which ordinarily penetrate into the brain but not to affect the permeability of those compounds which normally fail to enter the brain (Friedemann and Elkeles, '32; Broman, '38; Cooke, Hurst, and Swan, '42; Ôno, '44)

Cocaine and metrazol were included in the survey because of their primary neuronal effects and again because of their convulsive action. Although some of the higher doses of cocaine and metrazol caused convulsion, no effect on the brain-to-plasma ratios was noted. Bjerner et al. ('43, '44) and Campailla ('40), on the other hand, found metrazol seizures resulted in a permeability to the acid dye, trypan blue. Aside from the difference in the tracers used, this apparent discrepancy of results might possibly be explained on the basis of differences in the dosages used and corresponding differences in the relative severity of the effects produced. Our results, obtained for the most part in the subconvulsive dose range of the convulsive drugs used, are compatible with the view that within such dose ranges the action of these

agents is mainly neurogenic. Bjerner used metrazol in higher convulsive doses, which produced punctiform perivascular hemorrhages. Campailla's use of repeated metrazol seizures suggests a similar explanation.

Aminophylline (theophylline-ethylene diamine) caused a decreased sulfanilamide brain-to-plasma ratio. The experiment in which aminophylline, dehydration, and atropine sulfate were used in combination failed to produce a greater decrease in the sulfanilamide brain-to-plasma ratio than was obtained with aminophylline alone. These results again are difficult to compare with various studies reported in the literature. Cooke et al. ('42) found that theophylline-sodium acetate increased the brain permeability to the basic dye, methylene blue, but did not render the cerebral capillaries permeable to the acidic dye, trypan blue. Fröhlich and Mirsky ('42) observed that a combination of theophylline and acid fuchsin resulted in convulsions in adult rats. Although a synergistic effect may be involved, since acid fuchsin normally does not permeate the brain of adult rats they concluded that theophylline increased the permeability of the cerebral capillaries to this agent. The variation in results produced by theophylline upon various tracer compounds well demonstrates the complexity of permeability processes.

Sodium salicylate caused an increase in the sulfanilamide ratio of borderline significance. Rao ('33) noted that hydrotropic substances, such as sodium salicylate, increased the permeability of animal membranes to acids. Hanzlik ('12) found that sodium salicylate was effective in increasing the rate of passage of iodide across the wall of living, excised intestine.

Insulin showed no effect at a dosage level producing moderate hypoglycemia. Bjerner et al. ('43, '44) and Campailla ('40), on the other hand, found that trypan blue stained the brain when doses of insulin were used which produced coma. Such differences of observation on permeability effects are not surprising considering the differences in the dosage of

insulin and the physiologic effects produced, as well as the differences in the tracer molecules used.

Greig and various co-workers in a series of experiments have implicated the acetylcholine-cholinesterase system in permeability processes. Greig and Holland ('49) found that acetylcholine and physostigmine injected simultaneously into the dorsal lymph sacs of frogs greatly reduced the usual time required for acid fuchsin to permeate into the brain in convulsive concentrations. Greig and Mayberry ('51) observed that physostigmine decreased the time necessary for inducing anesthesia in mice by barbital or chloralose by one half. Chemical analysis of barbital levels showed that the effect in the case of barbital, at least, was due to an increased permeability rather than a synergism. Our results with acetylcholine and physostigmine are compatible with the view of Greig's group that the energy released by the enzymic hydrolysis of acetylcholine is important to permeability processes.

The term, "blood-brain barrier," has been used in this paper because it is unfortunately the convention to list studies concerned with many aspects of the variable permeation of substances from the blood stream throughout the brain under this title or a similar restrictive title, such as, "cerebrovascular permeability" or "the hematoencephalic barrier." These terms have been used in the literature without much regard to the degree of permeability involved and occasionally without adequate consideration or investigation of the locus of the semi-permeable membrane or membranes concerned. In fact, in the analyses of brain tissue as a whole, as in the present study, it is impossible to draw conclusions regarding the relative importance of any particular site or membrane in the permeability processes studied, except when compounds which do not normally enter the brain are used. Friedemann ('42) noted the undesirable negative connotation of the term, "barrier," to describe selective permeability, while Aird and Strait ('44) adduced evidence concerning the locus of a capillary endothelial barrier to which they suggested the term, "blood-brain barrier," be restricted. These authors

emphasized the necessity of a second permeability site, the cell membranes of nerve tissue proper, in explanation of the differences in the permeability of this capillary barrier as compared with the blood-CSF barrier. This viewpoint was supported by Onesti ('50). The importance now assigned to metabolic activity in transport processes (cf. Ussing, '53) leaves no doubt concerning the necessity of considering and differentiating the role of the nerve cell in explanation of the unique features of brain permeability.

The extensive use of acid dyes as indicators of permeability has lead to a somewhat biased view of cerebral permeability. These compounds have the advantage of providing rapid, visual evidence of changes in the permeability of the cerebral capillaries. However, the information is limited to qualitative changes, presumably of a rather drastic nature in the cerebral capillaries, since it concerns a class of compounds which ordinarily do not enter the brain at all. Such results are not necessarily applicable to other compounds of different characteristics, or metabolites, which, for instance, may freely diffuse across the capillary wall, but which are only selectively transported into or out of nerve cells.

SUMMARY

The effect of a number of drugs and measures upon the rate of passage of sulfapyridine and sulfanilamide into the brain of the rat has been studied. The greatest increase was noted after intense and probably traumatic staining with the supravital dye, trypan red, while little or no change occurred after moderate staining with this dye. Minor increases in the brain-to-plasma ratios of one or both sulfonamides were observed with the use of sodium bicarbonate, sodium nitrite, sodium salicylate, and physostigmine sulfate. Decreased ratios were observed with the injection of ammonium chloride, adrenalin, aminophylline, and acetylcholine and also following drastic dehydration. In general, all moderate measures, which were used in testing their possible effect on altering permeability, appeared to produce only minor changes.

LITERATURE CITED

AIRD, R. B., AND L. STRAIT 1944 Protective barriers of the central nervous system; an experimental study with trypan red. Arch. Neurol. and Psychiat., *51:* 54–66.

BJERNER, B., T. BROMAN AND A. SWENSSON 1943 Djurexperimentella Undersökningar över skador på blodhjärnbarriären vid schockbehandling. Nord. med., *17:* 469–471.

———— 1944 Tierexperimentelle Untersuchungen über Schädigungen der Gefässe mit Permeabilitätsstörungen und Blutungen im Gehirn bei Insulin-, Cardiazol- und Elektroschockbehandlung. Acta psychiat. et neurol., *19:* 431–452.

BROMAN, T. 1938 Om blodhjärn-och blodlikvorbarriarernas problem (Problem of hemato-encephalic barrier). Nord. med. tsdskr., *15:* 205–213.

BROMAN, T., AND A. M. LINDBERG-BROMAN 1945 An experimental study of disorders in the permeability of the cerebral vessels (''the blood-brain-barrier'') produced by chemical and physico-chemical agents. Acta physiol. Scandinav., *10:* 102–125.

CAMPAILLA, G. 1940 La barriera emato-encephalica nell-accesso cardiazolico e nello shock insulinico sperimentali. (Sulla colorazione vitale del sistema nervoso). Riv. di pat. nerv., *55:* 225–248.

COOKE, B. T., E. W. HURST AND C. SWAN 1942 Routes of entry into the nervous system of viruses introduced into the blood stream; experiments with the viruses of pseudorabies, herpes and infectious myxomatosis. Australian J. Exper. Biol. Med. Sci., *20:* 129–138.

FRIEDEMANN, U. 1942 Blood-brain barrier. Physiol. Rev., *22:* 125–145.

FRIEDEMANN, U., AND A. ELKELES 1932 Über auxoneurotrope Wirkung des Adrenalin und Hypophysin. Deutsche med. Wchnschr., *58:* 923–926.

FRÖHLICH, A., AND I. A. MIRSKY 1942 Susceptibility to convulsions in relation to age; influence of acid fuchsin on rats of various age groups. Arch. Neurol. and Psychiat., *47:* 30–37.

GEIGER, A., AND J. MAGNES 1947 The isolation of the cerebral circulation and the perfusion of the brain in the living cat. Am. J. Physiol., *149:* 517–537.

GOLDSWORTHY, P. D., R. B. AIRD AND R. A. BECKER 1954 The blood-brain barrier — the effect of acidic dissociation constant on the permeation of certain sulfonamides into the brain. J. Cell. and Comp. Physiol. *44* (3): 519–526.

GREIG, M. E., AND W. C. HOLLAND 1949 Increased permeability of the hemo-encephalic barrier produced by physostigmine and acetylcholine. Science, *110:* 237.

GREIG, M. E., AND T. C. MAYBERRY 1951 The relationship between cholinesterase activity and brain permeability. J. Pharmacol. and Exper. Therap., *102:* 1–4.

HANZLIK, P. J. 1912 Quantitative studies on the gastrointestinal absorption of drugs. II. The absorption of sodium iodide. J. Pharmacol. and Exper. Therap., *3:* 387–421.

HURST, E. W., AND O. L. DAVIES 1950 Studies on the blood-brain barrier. II. Attempts to influence the passage of substances into the brain. Brit. J. Pharmacol., *5:* 147–164.

MAGNES, J., AND A. GEIGER 1948 Effect of hydrogen and bicarbonate ions on the metabolic rate of the perfused cat brain. Federation Proc., *7:* 77.

ONESTI, S. J. 1950 The blood-brain barrier. McGill Med. J., *19:* 203–210.

ÔNO, H. 1944 The effect of the vegetative nervous system on the permeability of the blood-liquor and blood aqueous humor barriers at low pressure. (In Japanese only.) Nagoya Igakkai Zasshi, *59:*(2): 81–99.

PAPPENHEIMER, J. R. 1953 Passage of molecules through capillary walls. Physiol. Rev., *33:* 387–423.

PAPPENHEIMER, J. R., E. M. RENKIN AND L. M. BORRERO 1951 Filtration, diffusion and molecular sieving through peripheral capillary membranes; a contribution to the pore theory of capillary permeability. Am. J. Physiol., *167:* 13–46.

RAO, A. N. 1933 Beitrag zum Problem der Permeabilität und Permeabilitätsbeeinflussung. Biochem. Ztschr., *262:* 332–350.

RENKIN, E. M. 1952 Capillary permeability to lipid-soluble molecules. Am. J. Physiol., *168:* 538–543.

STERN, L., G. KASSIL AND E. LOKCHINA 1927 Effect de l'alcool et du CO sur le passage du bismuth du sang dans le liquide céphalo-rachidien. Compt. rend. Soc. de biol. (Paris), *97:* 648–650.

STERN, L., AND E. LOKCHINA 1927 Effet de l'empoissonnement par l'oxyde de carbone (gaz d'éclairage), l'hydrogène sulfuré et l'acide cyanhydrique, sur le fonctionnement de la barrière hématoencéphalique. Compt. rend. Soc. de biol. (Paris), *97:* 647–648.

TEORELL, T. 1949 Permeability. Ann. Rev. Physiol., *11:* 545–564.

TSCHIRGI, R. D., AND J. L. TAYLOR 1953 Influence of various agents on the blood-cerebrospinal fluid potential. Federation Proc., *12:*(1): 145–146.

———— 1954 A steady biolectric potential between the blood and the cerebrospinal fluid (CSF). Federation Proc., *13:* 154.

USSING, H. H. 1953 Transport through biological membranes. Ann. Rev. Physiol., *15:* 1–20.

NEURONAL METABOLISM AND ATP SYNTHESIS IN NARCOSIS [1]

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INTRODUCTION

The effects of narcotics are not limited to any one group of cells or tissues in the organism. However, most dramatic induced alterations of behavior appear consequent to the action of these diverse physical and chemical agents on the nervous system. That common physico-chemical mechanisms must be responsible for the induction of narcosis (as well as for recovery from it, — since one of its most interesting characteristics is its reversibility) is strongly suggested by the myriad structurally different compounds which are effective (alcohols, barbiturates, urethanes, etc.). It is of particular interest in relation to the study of changes in neuronal activity, that in common with anoxia and other similar nociceptive stressful states, narcosis stimulates (lowers the threshold?) before it depresses (raises the threshold?).

Many factors possibly associated with narcosis have been suggested as being the foci of these common mechanisms, among them the relative solubilities of various compounds in the lipoid and protein phases of the cell membrane, interference with respiratory enzymes, and blocking of oxidative breakdown and high energy phosphate bond production in the

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cell. Much work has been concerned primarily with the biochemical reactions in the cell which revolve around the utilization and synthesis of adenosine-triphosphate (ATP). It has been suggested (McLennan and Elliot, '51; Govier and Gibbons, '54), for example, that narcotics interfere with the direct acetylation process. Recent studies (Mendelson and Grenell, '54; Johnson and Quastel, '53) show that this is not the case. Again, some investigations appeared to indicate that the answer lay in either uncoupling of phosphorylation from oxidation (Brody and Bain, '54) or in direct suppression of the oxidative synthesis of ATP (Quastel, '43). The present series of experiments was designed to study the effects of chloretone and sodium pentobarbital on ATP synthesis and neuronal rate of oxygen consumption in vitro and in vivo.

METHODS

In vivo and in vitro experiments have been carried out. The former consisted of measurements of relative rates of oxygen consumption with the O_2 cathode of locally perfused cerebral cortical areas in the cat. In the in vitro experiments, respiration of homogenates of rat brain and heart muscle (ventricle) was measured in the Warburg apparatus. After equilibration, the narcotic was tipped into the tissue suspension. Aliquots of the whole suspension in the Warburg flasks were removed for ATP analysis after 90 minutes.

1. In vitro

(a) Tissue respiration: All samples of cerebral cortical tissue were taken from unanesthetized, adult, male white rats (a total of 60 experiments was performed). The animals were killed by decapitation following stunning by a blow on the head. The brain was removed rapidly and placed immediately in iced homogenizing solution. This solution consisted of: 0.04 M $MgCl_2$; 0.06 M KCl; 0.04 M Nicotinamide (the presence of this substance is imperative in order to prevent the breakdown of Coenzyme I). The pallium was dissected

as cleanly as possible away from the remainder of the cerebral hemisphere, quickly weighed and placed in a pre-iced tube containing three times the tissue wet weight of homogenizing solution. It was then ground for 90 minutes with a steel pestle. Of this 25 per cent homogenate, 0.5 ml was pipetted into an iced Warburg flask containing — in terms of concentration in the final reaction mixture — 0.02 M Na pyruvate and 0.05 M Phosphate buffer, pH 7.6. The contents of each flask totalled 3 ml (table 1). The center well of each flask contained KOH.

TABLE 1

Contents of Warburg flasks in milliliters

Na phosphate buffer, 0.05 M, pH 7.6	1.62 ml
Na pyruvate, 0.02 M	0.38 ml
25% rat homogenate cortex	0.5 ml
Exper. (pentob. or chlor.)	0.5 ml
Controls (buffer)	0.5 ml

Krebs solution for heart slices

NaCl	120 mM
KCl	2.4 mM
$CaCl_2$	1.7 mM
Phosph. Buffer $\{Na_2HPO_4, NaH_2PO_4$	0.02 M., pH 7.4
Na pyruvate	20 mM

The flasks were attached to the manometers, placed in the 37° C. water bath and allowed to equilibrate for 10 minutes. The flask side-arms contained distilled H_2O for the controls, and chloretone or Na pentobarbital in varying concentrations (see tables) for the experimentals. Two minutes were allowed to elapse before the first readings were taken following tipping in of the side-arm contents, in order to insure return of the flask contents to 37° C. All reactions were carried out in air. Readings were made at 30-minute intervals up to and including 90 minutes. The flasks were then removed and 1 ml samples of the suspension were pipetted into 3 ml of boiling H_2O. The tubes were allowed to cool, placed in the

deep freeze and later analyzed for adenosinetriphosphate (ATP).

For purposes of comparative study, samples of cardiac muscle (ventricle) were taken in the same way from similar unanesthetized, adult male rats. The hearts were removed while still pulsating and placed in iced Krebs solution through which 100% O_2 was bubbled. Slices of the ventricle were prepared, keeping the heart cold. The slices were rinsed in fresh, oxygenated Krebs solution, blotted and weighed on a torsion balance. They were then placed in previously prepared iced Warburg flasks containing Krebs solution and 0.02 M Na pyruvate. The flasks were equilibrated with 100% O_2 at 37° C. for 15 minutes. After equilibration, either Krebs solution (for the controls) or Na pentobarbital (prepared in Krebs solution) was tipped in and readings taken at 20-minute intervals for a total of 60 minutes. At this time the heart slice was removed and extracted in 3 ml of boiling water. After extraction the solution was frozen for future ATP analysis. The heart slice was dried at 60°C. overnight, and weighed. (The supernate remaining after removal of the heart slice from the Warburg flask was also analyzed for ATP).

(b) ATP determination: The method of ATP analysis is based on the linear luminiscence response of firefly extract to added ATP when all other factors are present in excess (Mg^{++}, luciferin and oxygen). A partially purified luciferase preparation was prepared from dried firefly lanterns according to a previously described procedure and was used throughout the present study (McElroy, '51a). The reaction mixture consisted of 0.2 ml luciferase, 0.5 ml luciferin, 0.1 ml 100 mM $MgSO_4$ and 0.05 M glycine buffer, pH 7.5, to give a final volume of 2.5 ml. Varying amounts of ATP were added to initiate light emission and the initial intensity was measured by means of a photomultiplier. The output of the cell was recorded by means of an Esterline-Angus automatic recorder. The unknown samples which were to be assayed for ATP were diluted appropriately and run in triplicate. The quantity

of ATP present in these samples was determined by comparison with the standard. Controls were always run to determine the effect of various drugs and reagents on the ATP recovery in the unknowns. The present assay is specific for ATP; other phosphorylated compounds have been shown to be completely inactive. The present procedure can detect 1 μg ATP per milliliter with an accuracy of approximately 5%.

2. *In vivo*

The techniques used in the in vivo experiments will be described very briefly, since a detailed discussion of all the problems involved is to appear in another paper (Davies and Grenell).

A small area of the cat's cerebral cortex (in this case, of the supra-sylvian gyrus), is perfused by means of a small glass cannula (150 μ in diameter at its tip) which is inserted into one of the pial arterioles. This vessel is tied off above and below the point of cannulation so that only selected side branches carry the perfusate into the surrounding cortex. The perfused area is usually about 5 mm in diameter. No arrest of circulation ever occurs in the area, and the preparations have remained healthy (as evidenced by response to stimulation) for as much as three hours if the perfusate contains washed red blood cells. Filtering the perfusion fluid through a 14-micron sintered glass filter will give a perfusate that is satisfactory for two hours even without the inclusion of the red cells.

The control perfusate has been an "incomplete" bicarbonate buffered Krebs solution, containing the following constituents:

Na^+	0.1432 M	HCO_3^-	0.0073 M
K^+	0.0059 M	glucose	0.0060 M
Ca^{++}	0.0025 M	O_2	95%
Mg^{++}	0.0012 M	CO_2	5%
Cl^-	0.1471 M	Knox gelatin	2%
$SO_4^=$	0.0012 M		(B-121)

The pH of the solution was 7.0 by test. The experimental perfusates consisted of the control solution plus Nembutal

in amounts calculated to give concentrations of the narcotic of 0.2 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 10.0 mM.

In addition, an adaptation has been used of the oxygen cathode for measuring local oxygen consumption at the cortical surface (Davies and Rémond, '47; Davies and Grenell; Grenell and Davies, '50a and b), with which oxygen consumption in the perfusion area may be measured at intervals. The principles underlying the operation of the oxygen cathode have been described elsewhere (Kolthoff and Lingane, '52; Davies and Brink, '42). The method of application here is discussed at great length by Davies and Grenell. With this method, local relative rates of oxygen consumption of cells in the perfused area were obtained before, during, and after perfusion with the nembutal solutions.

RESULTS

A. In vitro respiration and ATP

1. Rat cerebral cortex —

(a) Chloretone: The effects of 1–6 mM chloretone were measured on respiration and ATP content of rat cerebral cortex homogenate. The results obtained are shown in table 2 and figure 1. Averaging the results of 27 experiments, the lower concentrations of chloretone, i.e., one and 2 mM, in-

TABLE 2

Average effects of chloretone on respiration and ATP content of rat cerebral cortex homogenate
(27 experiments)

CHLORETONE CONCENTRATION	CONTROL RESPIRATION	EXPERIMENTAL RESPIRATION	% INHIBITION OF RESPIRATION	CONTROL ATP	EXPERIMENTAL ATP	% INHIBITION ATP
mM	*average*	*average*	*average*	*average*	*average*	*average*
6	157	45	71%	8.4	2.4	73%
4	165	89	46%	8.9	4.3	52%
2	156	127	19%	9.4	10.0	0%
1	163	156	4%	9.3	9.6	0%

Respiration in terms of $mm^3 O_2$/125 mg cortex wet wt./90 minutes.
ATP in terms of μg/3 ml total reaction mixture.

hibited the respiration only slightly without having any effect on the ATP content — other than what might be considered a slight *increase* (stimulation?). The respiration has been recorded in c mm O_2/125 mg cortical tissue wet wt./90 minutes. The ATP values are in μ gm/3 ml total reaction mixture. It is apparent that only high concentrations of the chloretone — i.e., 4 and 6 mM, which markedly inhibit respiration (46 and 71% inhibition respectively), affect the ATP concentration (52 and 73% respectively) severely. If a plot

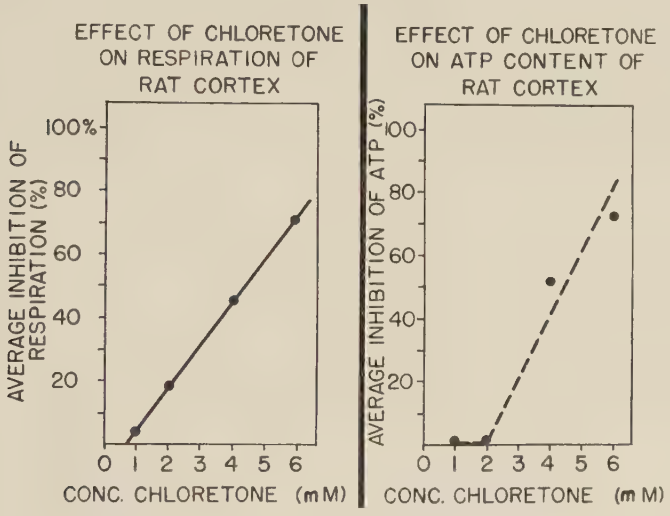

Figure 1

were made of the concentration of the narcotic against the ratio of the per cent inhibition of respiration to the per cent inhibition of ATP, it would show an almost 1 to 1 relationship at the 4 and 6 mM levels, dropping to 0 at the 1 and 2 mM concentrations. In other words, below a critical level no inhibition of net synthesis of ATP is observed.

(b). Sodium pentobarbital: Similar experiments were performed using sodium pentobarbital in place of chloretone. Inasmuch as the former is a much more potent narcotic, lower concentrations were employed (table 3; figure 2), rang-

ing from 0.1 to 3 mM. The figures shown in table 3 are the average of 26 experiments. No inhibition of respiration or ATP synthesis is evident with 0.1 mM pentobarbital, and only slight inhibition with 0.3 mM. The 0.5, 1, 2, and 3 mM concentrations all produce significant decreases in both respiration and ATP concentration. In essence, the results are quite similar to those seen with chloretone, except for the difference in range of effective concentration of the narcotic.

TABLE 3

Average effects of pentobarbital on respiration and ATP content of rat cerebral cortex homogenate
(26 experiments)

PENTOBARBITAL CONCENTRATION	CONTROL RESPIRATION	EXPERIMENTAL RESPIRATION	% INHIBITION OF RESPIRATION	CONTROL ATP	EXPERIMENTAL ATP	% INHIBITION ATP
mM	*average*	*average*	*average*	*average*	*average*	*average*
3	220	35.1	84%	17.7	2.3	86%
2	237	51.9	78%	21.8	4.1	81%
1	237	106	55%	21.8	12.6	42%
0.5	218	161	26%	20.5	15.9	22.6%
0.3	212	185	13%	20.8	17.4	16%
0.1	184	185	0%	8.17	12.3	0% (stim.?)

Respiration in terms of mm³ O_2/125 mg cortex wet wt./90 minutes.
ATP in terms of μg/3 ml total reaction mixture.

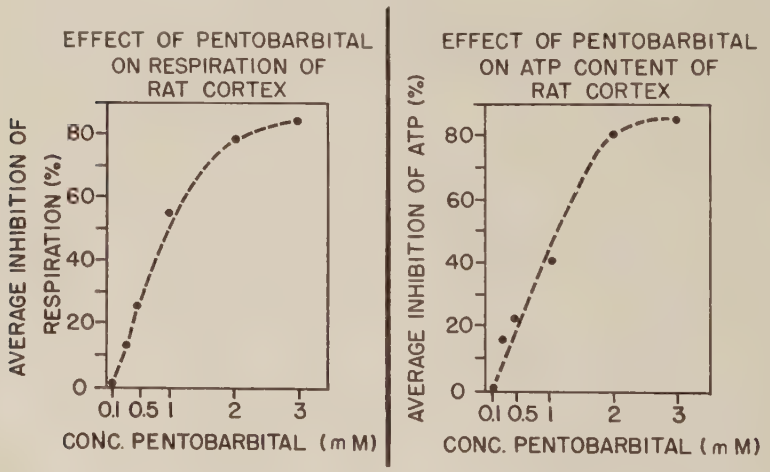

Figure 2

2. Rat ventricle

For purposes of comparison, the effects of sodium pentobarbital on cardiac muscle were examined. This tissue was chosen with the idea in mind that in a narcotized or anesthetized animal, the heart functions without interference, even though drastic changes in the nervous system may have taken place. In addition, the chemical system of muscle is one, of course, in which ATP is of the first importance. The rate of oxygen consumption of ventricle slices was recorded in

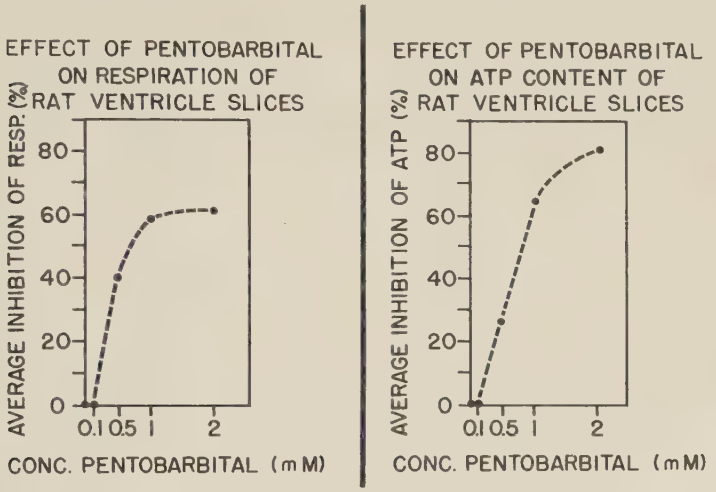

Figure 3

terms of micro-liters of oxygen per milligram of tissue dry weight per 60 minutes. The ATP values are recorded as micrograms per milligram of tissue, dry weight. The low concentration of 0.1 mM pentobarbital appears to give a slight increase in both respiration and ATP. Although the slope (shown in figure 3) of increasing ATP inhibition with increasing concentration of the narcotic seems to parallel that for respiration (for the lower concentrations), the figures in table 4 are interesting in several respects. It is clear that as with brain the low narcotic ('physiological') dose inhibits neither respiration nor ATP synthesis. In addition, it seems

that with the higher concentrations of the pentobarbital the ATP inhibition at first lags behind the inhibition of respiration (i.e., with 0.5 mM pentobarbital the inhibition of respiration is 40%, and of ATP 26%). As the concentration is raised further, a point is reached where both quantities are inhibited to an almost equal degree (1 mM pentobarbital) and above this level the cutback in ATP surpasses the interference with respiration (with 2 mM pentobarbital respiration is inhibited by 61% and ATP by 80%). It should also be noted that with the 1 and 2 mM concentrations of the narcotic a

TABLE 4

Average effects of pentobarbital on respiration and ATP content of rat ventricle slice
(8 experiments)

PENTOBARBITAL CONCENTRATION	CONTROL RESPIRATION Q_{O_2}	EXPERIMENTAL RESPIRATION Q_{O_2}	% INHIBITION OF RESPIRATION	CONTROL ATP	EXPERIMENTAL ATP	% INHIBITION ATP
mM	*average*	*average*	*average*	*average*	*average*	*average*
2	7.1	2.8	61%	0.51	0.10	80%
1	7.1	3.0	59%	0.51	0.19	62%
0.5	6.6	4.0	40%	0.68	0.50	26%
0.1	5.7	6.2	0%	0.67	0.83	0%

Respiration in terms of μl O_2/60 minutes/mg dry weight tissue.
ATP in terms of μg/mg dry weight tissue.

marked break in the slope of inhibition of respiration can be observed. Such marked leveling off is not seen with either the inhibition of ATP synthesis of the ventricle slices or the inhibition of respiration and ATP synthesis in the cerebral cortical homogenate.

B. In vivo respiration

Figure 4 shows the effects of pentobarbital (Nembutal) on the in vivo rate of oxygen consumption (as measured by the oxygen cathode) or a local area (supra-sylvian gyrus) of the cerebral cortex of the cat. Only a small drop in Q_{O_2} is observed with concentrations of the narcotic (in the perfusate)

below 0.5 mM. Two millimolar pentobarbital induced approximately 80% inhibition of oxygen consumption and a concentration of 10 mM reduced the Q_{O_2} to zero (Grenell and Davies, '50a).

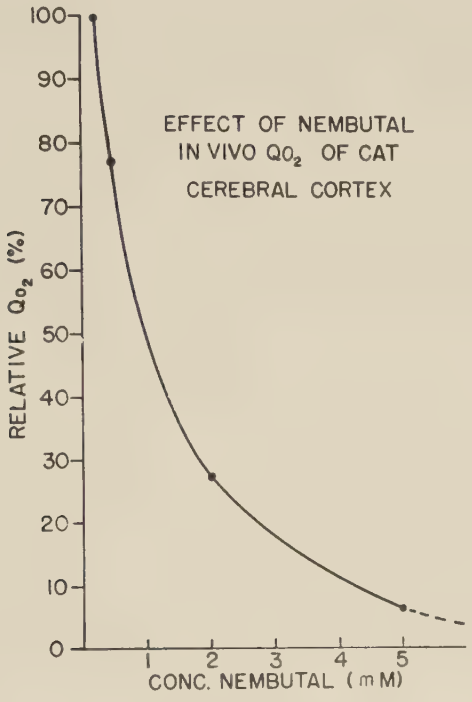

Figure 4

DISCUSSION

A wide variety of hypotheses are currently purporting to explain the fundamental mechanisms by which narcotics and anesthetics in sufficient concentrations are enabled to carry out a number of stimulatory, depressant, or inhibitory activities. Recent investigations have led to fairly wide-spread agreement that there must be a general basis for narcotic action on various types of organisms, cells, and processes. (In fact, there is a not inconsiderable amount of evidence indicative of common mechanisms for the several effects on nerve cells, for example, of many forms of 'stress' of the

nature of narcosis, anoxia, and gas poisonings.) The action of narcotics on nerve cells is not unique. As McElroy ('51b) has pointed out, ". . . the relative effectiveness of a particular series of compounds is independent of the cellular property studied be it inhibition of cell-divisions in sea-urchin eggs, sperm mobility, bioluminescence, photosynthesis or oxidative assimilation." Results obtained in many investigations suggest two (or possibly three, depending on the point of view) conceptual approaches to the factors responsible for this type of effective action — the 'physical' approach and the 'chemical'. The physical factors involve surface membrane and permeability phenomena; the chemical factors deal with inhibition of energy consuming reactions, e.g., the interference with cellular metabolism of the nature of inactivation of particular enzymes, or changes in labile phosphate esters with inhibition in the utilization of phosphate bond energy. On the basis of the data available at the present time, it would appear that the most logical view is what might be called 'physico-chemical'. By the use of this term it is suggested that physical effects of narcotics on the nerve cell membrane (Mullins, '54), for example, are associated with and may be forerunners of chemical alterations in the metabolic status of the neuron. These metabolic alterations, as pointed out above, may include a variety of reactions which themselves may influence such processes as membrane 'resynthesis' (e.g., production of high-energy bonds essential for formation and reformation of membrane lipoprotein molecules), and cell respiration — resting or in activity. It has been the intent of the present investigation to study some of the factors which might be presumed to be related to such chemical changes and consequently to shifts in neuronal behavior. More explicitly, the problems involved here relate to the following questions: Do the narcotics exert their effect by uncoupling crucial enzyme catalyzed reactions? Can it be said that the mechanisms of narcosis are based on the interference with oxidative processes revolving around the utilization and synthesis of adenosinetriphosphate (ATP)?

One of the ways of determining the validity of the theory of interference with neuronal metabolism has been to measure rates of oxygen consumption — in vitro and in vivo. Quastel and his associates demonstrated some years ago that many narcotics and anesthetics in sufficient concentration significantly inhibit in vitro oxygen consumption of cerebral tissue. These studies led to the general opinion that the depression of the Q_{O_2} was the primary action of these substances. This theory, however, has not stood up at all well when examined in the light of more recent data. Two points are of particular importance in this regard. The first is the problem of concentration of the narcotic. The second is concerned with the realization that the rate of oxygen consumption is not the primary factor to be dealt with. That is to say, that oxidative processes may be involved without being reflected by a change in rate of oxygen consumption and/or that several other possibilities exist which bear looking into, such as high energy P synthesis in anaerobiosis, group transfer and uncoupling. It is extremely unlikely from both the biological and chemical points of view, that all narcotic substances act on one specific enzyme path.

The question of the concentration of the narcotic is most vital. A good deal of the data upon which Quastel's views are based was obtained from experiments (Johnson and Quastel, '53a) in which unnecessarily high, 'unphysiological' concentrations of the drug were used. Recent results of the experiments of others (Larrabee et al., '52; Davies, '52; Grenell and Davies, '50a) as well as of those reported here, demonstrate that many narcotics and anesthetics in physiological, narcotic concentration do not inhibit respiration — i.e., may have slight or even no effect on the oxygen uptake of the brain. The results obtained in the present investigation are quite clear. Neither chloretone nor sodium pentobarbital depress the Q_{O_2} when they are present in low enough concentrations. Indeed, there are indications that low enough concentrations may even stimulate as Westfall ('51) has shown for phenobarbital. Both in vitro studies and those in vivo,

where perfusion of cerebral cortex with a solution containing increasing amounts of nembutal and chloretone (Davies, '52) has been carried out, show that the oxygen uptake can be markedly depressed by relatively enormous concentrations of these substances, but that in concentrations which all the available data would indicate to be those presumably close to the blood levels in narcosis no or very slight interference can be observed.

It was suggested above that other factors might be involved here. Many investigations have demonstrated (Jowett and Quastel, '37; Michaelis and Quastel, '41; Quastel, '43; Greig, '46; etc.) that narcotic agents might affect coupled reactions involving electron transfer. If so, as McElroy ('51b) has stated, ". . . such a system may become uncoupled as far as electron transfer and phosphate esterification is concerned by the process of reversible denaturation and yet leave intact the individual enzymes involved." Such changes would certainly influence the utilization and/or generation of energy by the system. Furthermore, our present knowledge of cellular metabolic systems is strongly indicative of the fact that available phosphate bond energy is of the utmost importance (Lipmann, '41; McElroy, '47; Lehninger and Friedkin, '49). That this is crucial is also demonstrated by the recent results of Larrabee et al. ('52), who report that ganglionic transmission is depressed markedly by narcotic concentrations which do not affect the Q_{O_2} and McIlwain ('53), who has shown that phenobarbital, in concentrations equal to the anesthetic level, inhibits the extra Q_{O_2} of *activity* of brain slices without any effect on the *resting* rate.

Consequently, since it is well known that ATP is one of the major sources of high energy phosphate bonds in the brain, it becomes essential to determine whether or not narcotic action (or even the inhibition of respiration) is based on interference with ATP utilization or synthesis. The evidence shows that with the lower concentrations of the narcotizing agents there is either a slight increase or no shift in the steady state level of ATP. Inhibition is seen only with

larger amounts of the narcotic. The measurements on cardiac muscle show the same thing, and in addition demonstrate a marked independence of ATP level and depression of the oxygen uptake. The major point, however, is that in no case does the net synthesis of ATP appear to be significantly depressed by the narcotic substances in reasonable concentration.

Since there are a large number of pathways by which ATP can be utilized it is obviously impossible to state, on the basis of present information, that the narcotics do not interfere with certain of these processes. The measurement of the steady state level of ATP in the presence and absence of the narcotic does not provide conclusive evidence on this point.

Recent studies by Mendelson and Grenell ('54) have shown quite clearly, however, that acetylation of sulfanilamide is not inhibited by a series of narcotics in massive concentrations. These in vitro experiments were performed in a system containing known amounts of ATP and Coenzyme A. In addition, Lu and Krantz ('53) found no significant influence on ATP dephosphorylation by various anesthetics and hypnotics. Bain ('52) and Brody and Bain ('54) have, on the other hand, demonstrated that various barbiturates inhibit oxidative phosphorylation, which should influence the ATP level, at least in certain parts of the cell. It is possible that at low, physiological concentrations of the narcotics oxidative phosphorylation as well as a pathway of ATP utilization are partially inhibited leading to an unchanged steady state level of ATP. Experiments are now under way to test this possibility. If it is true that oxidative phosphorylation is inhibited by the low narcotic concentrations while the overall ATP concentration is maintained at a normal level it suggests the possibility, if ATP level is important in narcosis, that the function of a particular cellular structure is important insofar as narcotic action is concerned.

Numerous physiological and biochemical studies suggest indirectly that the important structures which may be effected during narcosis are the mitochondria. Brody and Bain's studies support the general idea that the synthetic processes

associated with the mitochondria are sensitive to the narcotics. It is possible that in vivo the mitochondria activity may be depressed without an overall change in the ATP concentration becoming immediately apparent. This would depend a great deal on the rate and equilibrium conditions between ATP and other forms of organic phosphate including that bound to the gel structures. It is important that in oxidative phosphorylation problems a system, such as hexokinase and glucose, is added to accelerate any changes in phosphate turnover. It is only under these conditions that the effect of the narcotics can be demonstrated with the mitochondrial preparations. This may also have a bearing on the sensitivity of "resting" versus "active" tissues to these various agents.

Another important point that should be emphasized in this respect is that phosphate bond energy represents only one type of biological bond energy. In suggesting earlier that phosphate bond energy may be important in narcotic action it was assumed that phosphate bond energy was in equilibrium with and reflected the level of other forms of bond energy. Recent studies on group transfer have clearly shown that phosphate is not necessarily an obligatory component and in fact may be separated structurally from those systems which would immediately equilibrate these other energy-rich groups with phosphate bond energy. Thus narcotics may interfere, in time, with phosphorylation processes but they may be doing so by affecting group transfer processes in which the energy of the group would be lost as heat. The speed with which they would be reflected in the ATP level would depend upon structural and other considerations. Experiments are now in progress to test the effect of narcotics on other group transfer processes and whether the narcotics influence these processes by a physical alteration of the cellular structures which are essential for these coupled reactions.

CONCLUSIONS

Our experiments have produced no convincing evidence that the net synthesis of ATP is a crucial factor in the basic

mechanisms of the general action of narcotic agents. In addition, both in vitro and in vivo experiments fail to substantiate the theory that the primary action of narcotics is on the rate of oxygen uptake. These results give us no reason to believe that the mechanisms of narcosis are based on interference with the steady state level of ATP. To be sure, evidence for interference with such mechanisms is quite clear when the narcotic substances are present in the system in extremely high concentration. The fact, however, that this is an obviously unphysiological situation, makes it necessary to reject this reaction as the basic one.

This does not mean that alterations in cellular metabolism are not fundamentally involved in narcosis. It merely implies that the crucially related steps must be sought for elsewhere — perhaps on a slightly different basis. Of several factors which could be the common path for all types of narcotic action, only one possibility has been eliminated.

SUMMARY

A series of experiments was undertaken to determine the role of possible inhibition of the synthesis of a high energy phosphate bond (i.e., ATP) in the effective action of narcotic agents. Quantitative measurements were made of the rates of oxygen consumption of rat and cat cerebral cortex — the former in vitro with the Warburg technique, and the latter in vivo with the oxygen cathode. In the Warburg experiments on rat cortex homogenate, simultaneous quantitative determinations of ATP were carried out in all cases; both in the presence and absence of various concentrations of chloretone and sodium pentobarbital.

It is concluded from the results of these experiments that these narcotics in concentrations which would presumably exist in the tissue during a period of effective narcosis, do not significantly inhibit either the resting rate of oxygen uptake or the rate of net synthesis of adenosinetriphosphate. Consequently, the depression produced by such substances must be related to other mechanisms.

[R. G. Grenell]

LITERATURE CITED

BAIN, J. A. 1952 Enzymatic aspects of barbiturate action. Fed. Proc., Am. Soc. Exp. Biol., *11* (No. 2): 653–658.

BRODY, T. M., AND J. A. BAIN 1954 Barbiturates and oxidative-phosphorylation. J. Pharm. Exp. Therap., *110:* 148–156.

DAVIES, P. W. 1952 Effect of chloretone on oxygen consumption and negative spike response of cerebral cortex. Fed. Proc., Am. Soc. Exp. Biol., *11:* 30.

DAVIES, P. W., AND F. BRINK 1942 Microelectrodes for measuring local oxygen tension in animal tissues. Rev. Sci. Instr., *13:* 524–533.

DAVIES, P. W., AND R. G. GRENELL Metabolism and function in the cerebral cortex under local perfusion, with the aid of the oxygen cathode for measuring surface Q_{O_2}. (To be sent to press.)

DAVIES, P. W., AND A. RÉMOND 1947 Oxygen consumption of the cerebral cortex of the cat during metrazol convulsion. Chapter XIV in *Epilepsy,* Vol. XXVI, Res. Publ. Ass. nerv. ment. Dis., 205–217.

GOVIER, WILLIAM M., AND A. J. GIBBONS 1954 Pentobarbital inhibition of sulfanilamide acetylation in pigeon liver extracts. Science, *119:* 185–186.

GREIG, M. E. 1946 The site of action of narcotics on brain metabolism. J. Pharm. Exp. Therap., *87:* 185–192.

GRENELL, R. G., AND P. W. DAVIES 1950a Effect of nembutal on the in vivo rate of oxygen consumption of the cerebral cortex. Trans. Am. Neur. Assn., 105–108.

GRENELL, R. G., AND P. W. DAVIES 1950b Respiration of cerebral cortex in vivo, in the absence of glucose. Fed. Proc., Am. Soc. Exp. Biol., *9:* 52.

JOHNSON, W. V., AND J. H. QUASTEL 1953a Narcotics and biological acetylations. Nature, *171:* 602–605.

———— 1953b A comparison of the effects of narcotics and of 2,4,-dinitrophenol on sulfanilamide acetylation. J. Biochem., *205:* 163–171.

JOWETT, M., AND J. H. QUASTEL 1937 The effects of narcotics on tissue oxidations. Biochem. J., *31:* 565–578.

KOLTHOFF, I., AND J. J. LINGANE 1952 Polarography, Interscience Publishers, New York.

LARRABEE, M. G., J. G. RAMOS AND E. BULBRING 1952 Effects of anesthetics on oxygen consumption and on synaptic transmission in sympathetic ganglia. J. Cell. and Comp. Physiol., *40:* 461–494.

LEHNINGER, A. L., AND M. FRIEDKIN 1949 Esterification of inorganic phosphate coupled to electron transport between dehydrodiphosphopyridine nucleotide and oxygen. J. Biol. Chem., *178:* 611.

LIPMANN, F. 1941 Metabolic generation and utilization of phosphate bond energy. Adv. Enzymol., *1:* 99–162.

LU, G., AND J. C. KRANTZ, JR. 1953 Anesthesia XXXIX: The in vitro studies of the influence on ATP dephosphorylation by central depressants and stimulants. Anesth., *14:* 348–358.

McELROY, W. D. 1947 The mechanism of inhibition of cellular activity by narcotics. Quart. Rev. Biol., *22:* 25–58.

McElroy, W. D. 1951a Properties of the reaction utilizing adenosinetriphosphate for bioluminescence. J. Biol. Chem., *191:* 547–557.

——— 1951b The reversible denaturation of enzymes by narcotics. Mécanisme de la Narcose, CNRS, Paris, 89–103.

McIlwain, H. 1951 Metabolic response in vitro to electrical stimulation of sections of mammalian brain. Biochem. J., *49:* 382–393.

——— 1953 The effect of depressants on the metabolism of stimulated cerebral tissues. Biochem. J., *53:* 403–411.

McLennan, H., and K. A. C. Elliott 1951 Effects of convulsant and narcotic drugs on acetylcholine synthesis. J. Pharm. Exp. Therap., *103:* 35–43.

Mendelson, J., and R. G. Grenell 1954 Effects of narcotics on direct acetylation of sulfanilamide. Science, *120:* 802–803.

Michaelis, M., and J. H. Quastel 1941 The site of action of narcotics in respiratory processes. Biochem. J., *35:* 518–533.

Mullins, L. J. 1954 Some physical mechanisms in narcosis. Chem. Rev., *54:* 289–323.

Quastel, J. H. 1943 Effects of narcotics and benzedrine on metabolic processes in the central nervous system. Trans. Faraday Soc., *39:* 348–359.

Westfall, B. A. 1951 Effects of pentobarbital, amobarbital and barbital on oxygen consumption of brain slices. J. Pharm. Exp. Therap., *101:* 163–166.

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